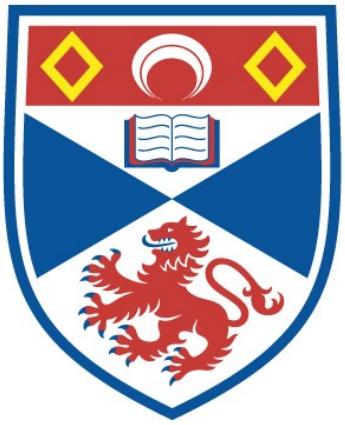
THE ECOLOGICAL SIGNIFICANCE OF HETEROTROPHY IN THE CARBOHYDRATE METABOLISM OF INTERTIDAL BROWN ALGAE

David Charles Jackson

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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The Ecological Significance of Heterotrophy in the Carbohydrate Metabolism of Intertidal Brown Algae.

by

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Thesis presented for the degree of Doctor of Philosophy.

Gatty Marine Laboratory,
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January, 1971.

DECLARATION

I hereby declare that the following thesis is based upon work done by me, that the thesis is my own composition, and that it has not been previously presented for a higher degree.

The research was carried out at The Gatty Marine Laboratory, University of St. Andrews, under the supervision of Dr. E. A. Drew.

CERTIFICATE

I certify that David C. Jackson has spent twelve terms of research under my direction, that he has fulfilled the conditions of Ordinance General No. 12 and Resolution of the University Court 1967, No. 1, and that he is qualified to submit the accompanying thesis in application for the degree of Doctor of Philosophy.

Curriculum vitae

I was born in Oldham, Lancashire, in 1943, and was educated at Chadderton Grammar School, and St. Andrews University, Scotland. In 1966 I graduated with second class honours (2) B.Sc. in Botany. I was awarded the degree of M.Sc. in 1967 for a thesis entitled "Nuclear Phenomena in *Sphacelaria britannica* Sauv.". Thereafter, I held a SRC post-graduate studentship of three years duration, in which time the practical work of the following dissertation was completed.

Abstract

Carbohydrate metabolism and heterotrophic carbon fixation have been studied in the intertidal macrophytic brown algae Pelvetia canaliculata and Fucus spiralis. In neither of these species can the heterotrophic metabolism of glucose support net growth. Photosynthetic rate, however, is enhanced by over 40% in Pelvetia in the presence of exogenous glucose. It is postulated that this glucose lowers the light requirement for photosynthesis rather than acting as a substrate for significant levels of heterotrophic carbon fixation.

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

Most investigations of the nutrition and metabolism of algae have, to date, been carried out using unicellular species. Only in recent years has attention been directed to macroscopic algae. Although these algae conform with the patterns established for autotrophic nutrition in the microscopic forms, albeit often with very different storage carbohydrates, their heterotrophic growth is still poorly understood, and available information inconclusive.

The subject of heterotrophic nutrition has been reviewed by Skinner and Gardner (1930), Myers (1951), Fogg (1953), Pringsheim (1959), and Danforth (1962). Heterotrophy has been variously defined to mean either an obligate process for the aquisition of exogenous amino acids, vitamins and 'growth factors' which the alga is unable to synthesize (Fogg, 1953); or, more loosely, a facultative dark assimilative mechanism for the accretion of exogenous organic carbon (in sensu Danforth, 1962; Bidwell and Ghosh, 1962, 1963; Drew, 1969). The latter interpretation, which is equivalent to the facultative chemo-organotrophy of Fogg (1953), does not imply heterotrophy and autotrophy to be mutually exclusive in the provision of any given metabolite, and thus either or both processes may be

simultaneously operative. Furthermore, any product of heterotrophic nutrition so defined, might under favourable conditions also be produced autotrophically. Although most pigmented algae grow under favourable environmental conditions (by means of autotrophic nutrition) it is becoming increasingly apparent that photosynthesis alone may often be inadequate for net growth to take place. Many algae, at least under laboratory conditions, appear able to overcome this problem by the facultative utilization of exogenous organic substances, thus enabling growth to take place in darkness and/or in the absence of carbon dioxide.

Those substrates which can be utilized for heterotrophic growth in culture experiments are shown in table 1:1. The picture is almost certainly incomplete. In certain instances the substrate indicated is the only one which has been tried (see, for example, Himanthalia et seq., Drew, 1969), whereas in other cases heterotrophic growth has been exhaustively studied, and yet still only one substrate is utilised (e.g., Navicula pelliculosa, Lewin, 1953).

In general, autotrophy and heterotrophy have been considered to act in such a way that the latter compensates only for slight deficiency in the former, but is usually unable to enhance maximal growth rate achieved under optimal conditions for photosynthesis (Roach, 1928, working on

Table 1:1. Utilization of exogenous metabolites by algae.

Species	Substrate	Reference
CHLOROPHYCEAE		
Chlorella vulgaris	G,F,Gal,C,L.	Neish (1951), but c.f. Finkle et al. (1950).
C. vulgaris "Wann" str.	Krebs cycle acids.	Eny (1950, 1951).
C. pyrenoidosa	G,Gal, acetate.	Samejima & Myers (1958).
C. ellipsoidea	G,Gal,ac,gly.	"
Scenedesmus sp. D3 str.	G,F,Gal,M,L,Ma,S.	"
S. quadricauda	G,F,M,Gal,Sor.	Taylor (1950, 1960a).
Prototheca zopfii (N.P.)	G,F,Gal,M,Gl,D,Fa,al.	Barker (1935).
"	pyruvate, lactate	Anderson (1945).
Polytomella coeca (N.P.)	Krebs acids,Fa,al.	Lwoff et al. (1949, 1950), Wise (1955, 1959).
Polytoma uvella (N.P.)	butyrate, caproate	Cirillo (1955, 1956, 1957).
Trebouxia humicola	F.	Roach (1926, 1927).
CRYPTOPHYCEAE		
Chilomonas paramecium (N.P.)	Krebs acids,Fa,al.	Cosgrove & Swanson (1952), Holz (1954).
EUGLENOPHYCEAE		
Euglena gracilis "Vischer" str.	acetate, butyrate.	Cramer & Myers (1952).
E. gracilis var. bacillaris	G,Krebs acids,Aas.	"
XANTHOPHYCEAE		
Tribonema aequale	G,S,ac,citrate.	Belcher & Fogg (1958).
T. minus	G.	"

Table 1:1. (continued)

Species	Substrate	Reference
BACILLARIOPHYCEAE		
Navicula pelliculosa	G.	Lewin (1953).
PHAEOPHYCEAE		
Fucus vesiculosus	G, Mannitol.	Bidwell & Ghosh (1962, 1963)
	G, Mann, Gal, F, M.	Drew (1969)
Ascophyllum nodosum	G, Mann, Gal, F, M.	"
Pelvetia canaliculata	G, Mann, Gal, F, M.	"
Himanthalia elongata	Glucose	"
Chorda filum	"	"
Cystoseira granulata	"	"
Halydris siliquosa	"	"
Laminaria digitata	"	"
L. saccharina	"	"
Pylaiella littoralis	"	"
Colpomenia sinuosa	"	"

Key. Aas amino acids; ac acetate; al alcohols; C cellobiose; D dihydroxyacetone; F fructose; Fa fatty acids; G glucose; Gal galactose; Gl glycerophosphate; gly glycollate; L lactose; Ma mannose; Ma maltose; Mann mannitol; N.P. non-photosynthetic

Algal classification is according to Parke and Dixon (1964).

Scenedesmus costulatus var. chlorelloides; Myers, 1951, working on Chlorella pyrenoidosa). This has been attributed to the ability of photosynthesis to saturate with its products the synthetic systems involved in growth, so that under optimum conditions for photosynthesis some factor other than the rate of carbon assimilation limits the rate of growth (Fogg, 1953).

Enhanced maximal carbon accretion and growth due to heterotrophy in the light has been refuted by Myers (1951) on the basis of incomplete CO_2-saturation of photosynthesis. However, Bunt (1969) found that in the marine diatom Cocconeis (species unidentified), heterotrophic uptake of exogenous organic metabolites takes place only in the light. Ochromonas malhamensis, the holozoic chrysophyte, is a particularly well documented instance of increased growth due to heterotrophic nutrition, even at saturating light intensity (Hutner et al., 1953, 1957; Reazin, 1954, 1956; Pringsheim, 1955; Myers and Graham, 1956; Vishniac and Reazin, 1957; Aaronson and Baker, 1959), and it has been suggested that organic substrates can act not only as sources of carbon but also as H-donors for photoreduction (Vishniac and Reazin, 1957).

Obligate autotrophs have a limited range of habitable niches available to them, and the same is true of the apochlorotic obligate heterotrophs. However, the facultative

heterotrophs, by virtue of their metabolic plasticity, are well adapted physiologically to cope in a variety of environmental conditions. The ability to grow in darkness or diminished light by utilizing substrates of high chemical potential energy such as sugars, amino acids, alcohols, fatty acids and Kreb's cycle intermediates has obvious ecological advantage, particularly for those algae inhabiting soils and polluted waters, and possibly also for arctic algae growing beneath thick ice.

Prompted by the work of Kjellman (1883) and Lund (1958), Wilce (1967) has offered an hypothesis "that heterotrophy bolsters photosynthesis in arctic deep water benthic marine algae". The hypothesis remains unsubstantiated concerning the heterotrophic potentials of the algae; nor has an exogenous carbon source been detected in that environment. Since prolonged dark-season dormancy is apparently absent in arctic algae (Kjellman, 1883) the hypothesis does seem plausible, especially if one considers also the work on Chlorella by Fogg and Belcher (1961) and Nalewajko et al. (1963), which indicates the efficiency of heterotrophic uptake of glycollate to be enhanced at low light intensity. Data offered by Wilce (1967) in support of his hypothesis will be discussed at greater length later.

Much of the work described above has involved laboratory

experiments in which metabolites have been added to the incubation medium. In this way heterotrophic potentials of various species (table 1:1) have been determined. But what of the occurrence of these metabolites in vivo?

The major sources of organic compounds in natural waters have generally been assumed to be detritus, cell debris, and the excretions of phytoplankton and zooplankton, but the relative importance of these sources is unknown, and will probably show some regional variance. Although the benthic seaweeds may have standing crops vastly greater than those of phytoplankton (Blinks, 1955), they have largely been ignored as a possible source of dissolved organic matter (Strickland, 1965, but see Sieburth, 1969b).

Soluble organic carbon in the North Sea and North Atlantic has been estimated at 1-5 mg/l (Duursma, 1961; Menzel and Vaccaro, 1964). Plunkett (1957) has found several free sugars (including sucrose, glucose, fructose, galactose, arabinose and zylose) in deep-sea sediments, and Koyama & Thompson (1959) have demonstrated the presence of acetic, formic, lactic and glycollic acids in Pacific water. It is suggested that such carbon compounds are excreted by algae (Fogg, 1959), or come from dead cells and detritus (Duursma, 1961). There is much evidence that environmental factors such as light, temperature and emersion influence this excretion (Fogg, 1963, 1966; Hellebust, 1965; Sieburth, 1969b).

A number of different compounds have been found to be excreted by algae. Glycollic acid was shown to be an excretory product of photosynthetically active phytoplankton (Tolbert and Zill, 1956; Nalewajko et al., 1963; Fogg and Nalewajko, 1964). The loss may account for up to 80% of the total carbon fixed. Algal productivity and the excretion of organic substances by algae have been reviewed by Fogg (1958, 1963 and 1966).

Sieburth (1969b), working on Chondrus crispus, Ascophyllum nodosum, Fucus vesiculosus, Laminaria digitata, and L. agardhii, found carbohydrate to be the major exudate, along with smaller amounts of nitrogenous and polyphenolic compounds. The loss by Fucus, which was coupled directly to photosynthesis, accounted for approximately 40% of the net carbon fixed daily. Thus, the importance of benthic seaweeds in the provision of dissolved organic carbon in the sea should not be overlooked. It is possible that the large populations of annual benthic species, along with the extensive blooms of algal unicells, will prove to be the source of organic carbon postulated to be present in arctic waters by Wilce (1967).

Johannes and Webb (1965) found various amino acids, always including arginine and taurine, to be released by zooplankton. They estimate that these amino acids represent

22 and 25% of primary organic production in the Sargasso Sea and the Gulf Stream respectively. Zooplankton excretions are also a source of organic and inorganic phosphates, although the exact chemical identity of such phosphates is unknown (Pomeroy et al., 1963). Butler et al. (1970) have found that Calanus excretes increased amounts of organic phosphate during the spring diatom bloom, whilst organic nitrogen excretion remains at a constant low level throughout the year.

In addition to soluble organic compounds there are the organic aggregates of humic material or Gelbstoff, which appear to be precipitated in the sea as complexes of polyphenolic compounds with proteinaceous and carbohydrate material (Sieburth, 1969a). Unless the precipitation is reversible, for example in waters of depleted organic content, it is unlikely that such aggregates could be used directly as substrates for heterotrophic nutrition. Extracellular proteinase has, however, been detected from certain unicells (Roach, 1926; Pringsheim, 1951), but the occurrence of such enzymes in algae is likely to be uncommon.

Another factor of considerable importance, at least in coastal waters, is sewage, which on decomposition liberates high concentrations of fatty acids and alcohols. The concommitant low light penetration in such water might

necessitate at least some heterotrophy for the photosynthetic algae known to be living there.

Organic metabolites are apparently present in natural waters in sufficient quantities for heterotrophic nutrition to seem a plausible hypothesis not only in arctic waters but wherever conditions of low light intensity prevail. Should this prove to be true, the importance of such a feed-back system of high chemical potential energy cannot be overstated.

For intertidal algae a further factor, emersion, detrimentally affects photosynthesis. It has been demonstrated by Bidwell and Craigie (1963) that photosynthesis can proceed at maximal efficiency for only a few hours each day in these algae, although Sieburth (1969b) has demonstrated some photosynthesis in emersed intertidal algae moistened by rain-water. Sieburth has further shown that up to 40% of the net carbon fixed photosynthetically by F. vesiculosus during immersion is lost by exudation into the sea. And yet the very existence of this and other intertidal species is evidence of their ability to cope under these conditions. How they do so is the underlying theme of the work which follows. It may be necessary to extend the hypothesis of Wilce to include intertidal algae; the possibility seems particularly plausible for algae living in those regions

such as sewage outfalls where the very agent which diminishes light penetration is also a source of available organic carbon.

Previous work concerning heterotrophy in intertidal algae has shown considerable species variation in the uptake patterns of exogenous carbohydrates and their subsequent metabolism (Bidwell and Ghosh, 1963; Drew, 1969). Although these algae have been included in table 1:1 it is by no means clear that heterotrophy will sustain their growth.

The macrophytic brown algae Pelvetia canaliculata and Fucus spiralis were chosen as the experimental material for the present investigation. This choice was made for two reasons; firstly, both are intertidal species, and yet marked differences in their heterotrophic metabolism have been demonstrated (Drew, 1969); secondly, because they are upper littoral species, and hence are emersed for considerable periods of time, it is conceivable that a possible need for heterotrophic supplement might be most readily demonstrable in them.

Although this is the basis of the research, other (non-heterotrophic) mechanisms may be postulated to supplement photosynthesis in these algae. Decreased respiratory rate during emersion, and hence conservation of respiratory substrate, is one such means (as was demonstrated by Bidwell and Craigie, 1963, working on

F. vesiculosus). Alternatively, the photosynthetic process might be so adapted that heterotrophic supplement is not required, even though the useful photoperiod is short; this might be accounted for in terms of high photosynthetic efficiency or low compensation point.

Thus the research involves the nature and quantitative importance of heterotrophy in Pelvetia and F. spiralis, and the possible requirement for this process in these intertidal algae. The line of investigation has included analysis of endogenous carbohydrates and their respiratory metabolism, the uptake and metabolism of exogenously supplied glucose, the possibility of mass translocation of metabolites, and estimation of the pentose-phosphate pathway. Also, heterotrophic carbon fixation has been compared quantitatively to fixation by other methods, and finally, a critical assessment has been made of Wilce's hypothesis.

CHAPTER 2

MATERIALS AND METHODS

Contents

MATERIALS

1. Sources and preparation of algae

Pelvetia canaliculata and Fucus spiralis were collected from the Castle Sands, the Kinkell Rocks, or the Step Rock, St. Andrews, on descending tides the day before use in experiments. They were kept in darkness at 5°C, and prior to use were thoroughly washed in filtered sea-water. Algae contaminated by epiphytes were discarded.

2. Reagents

'Analar' or Laboratory Reagent chemicals from British Drug Houses were used throughout, except as indicated below.

Adenosine 5' -triphosphate, adenosine 5' -diphosphate, nicotinamide-adenine dinucleotide phosphate (mono-sodium salt), crystallized and lyophilized bovine serum albumin (BSA), and glucuronic acid, were obtained from the Sigma Chemical Company.

Reduced nicotinamide-adenine dinucleotide phosphate (tetra-sodium salt) was kindly provided by Dr. S. Bayne (Department of Biochemistry, St. Andrews University), and was a Boehringer product.

Radioactive chemicals were obtained from the Radiochemical Centre, Amersham. Isotopes used were sodium bicarbonate-^{14}C, D-glucose-U-, -1-, and -6-^{14}C, and

D-mannitol-1-^{14}C. None were found to be at variance with the manufacturer's claimed specifications.

METHODS

Experimental Procedures

1. Physical conditions of experiments

(a) Osmotic pressure (O.P.) of incubation media

When the salinity of sea-water is 35‰ (35gm salt per kg sea-water) the O.P. is 22.5 atmospheres at N.T.P. (Sverdrup et al., 1949). Experiments were generally conducted at 10°C, at which temperature the O.P. of such sea-water is 23.3 atmospheres.

Glucose administered exogenously at a concentration of 0.2mM would exert an O.P. of 0.0048 atmospheres at 10°C, this representing a change in O.P. of less than 0.002%. This is well within the range of variability to be found in the sea (salinity 33-37‰, representing approximate O.P. values of 22 and 25 atmospheres respectively at 10°C). Addition of substrates at such concentrations should therefore have no adverse direct effects on the plant material.

(b) Sea-water pH and buffer effect

Sea-water contains carbonic and boric acids and their salts and is thus a buffer solution. The following equilibria represent only the carbonic acid system, but that for boric

acid is essentially similar.

$$2H^+ + CO_3^{=} \longleftrightarrow H^+ + HCO_3^- \longleftrightarrow H_2CO_3 \longleftrightarrow CO_2 + H_2O$$

The buffer capacity, or susceptibility to pH change on the addition of strong acid or alkali, was found to be inadequate in experiments in which the tissue volume was substantial relative to the volume of sea-water incubation medium. Thus, at 10°C in darkness, 7 ml. of sea-water (pH 7.9) containing 250 mg. fresh weight of respiring Fucus vesiculosus apices attained a pH of 6.75 after 20 hours in a sealed flask. Since pH = $-\log(H^+)$ this represents more than a tenfold increase in hydrogen ion concentration, and is attributed to a respiration induced increase in p_{CO_2}, which tends to shift the equilibrium shown above to the left.

Temperature flux is also of prime importance when considering buffer systems. Laboratory sea-water was kept at room temperature (about 20°C) whereas experiments were normally run at 10°C. This decrease in temperature caused a decrease in p_{CO_2} and hence increased pH. Thus, after quickly cooling sea-water from room temperature to 10°C, pH changed with time as shown in figure 2:1.

It was required to measure CO_2 released in respiration. A sealed system containing a small phial of KOH was used; KOH, by producing a CO_2-free "atmosphere", tended to partially offset the pH decrease caused by respiration (flask a., tabb

Figure 2:1. The relationship of pH with time after rapid cooling of sea-water to 10°C.

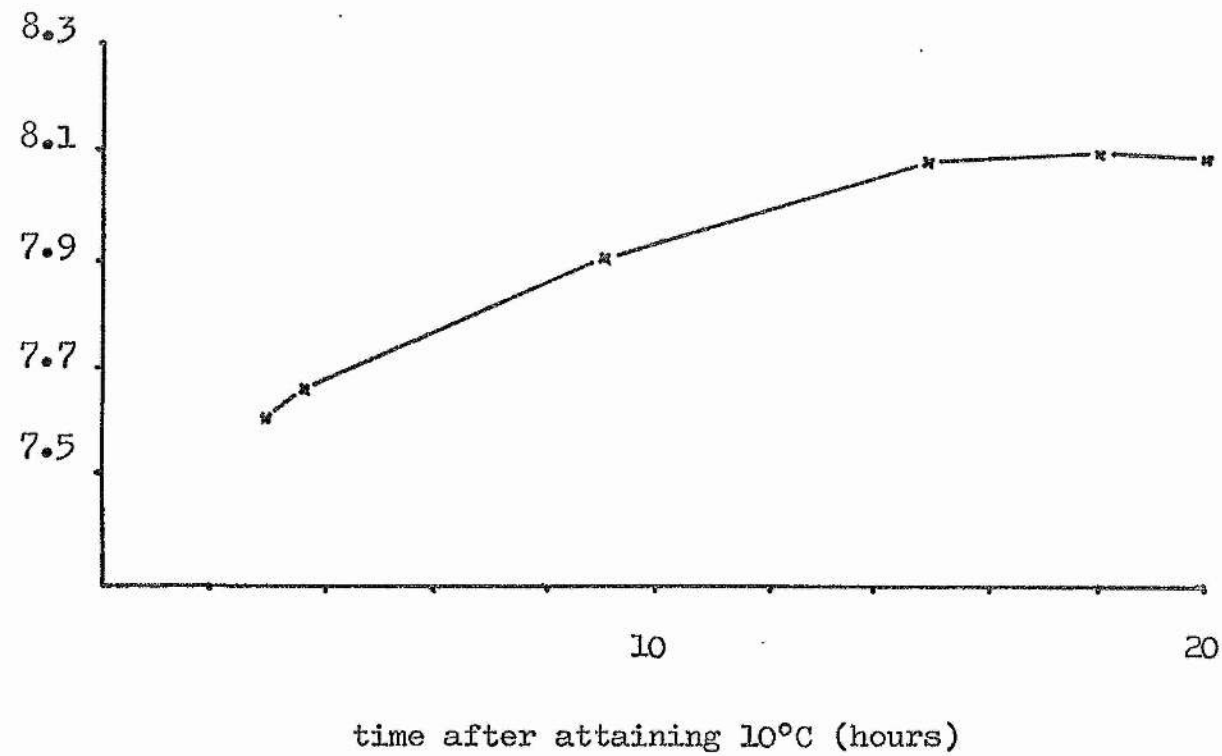


Table 2:1. The effect of respiration on the pH of incubation media. Flasks were sealed and contained 2ml. 10% KOH in small phials. Incubation time 24 hours at 10°C. Tissue weight 200 mg. fresh weight of *Fucus spiralis*. Incubation medium 3ml. sea-water.

Flask a. p_{CO_2} equilibrated sea-water.

Flask b. non-equilibrated sea-water.

Flask c. p_{CO_2} equilibrated sea-water, including 10mM Tris-HCL buffer.

Flask	Initial pH	Final pH	pH change
a	8.09	7.79	-0.30
b	7.73	7.65	-0.08
c	8.17	8.13	-0.04

2:1). This effect was enhanced when sea-water was not allowed to equilibrate at 10°C prior to use as the incubation medium (flask b., table 2:1).

In an attempt to further minimise pH change due to respiration, Tris (hydroxymethyl) amino methane was used as a buffer. The choice of Tris was made for two reasons. Firstly, it is not used as a carbon source, at least by green algae (Wiedeman, 1964); and secondly having pK_a 8.07 (West and Todd, 1963), which is close to the pH of sea-water, its buffer capacity is maximal at the pH of these experiments. 10mM Tris-HCl buffered sea-water was used in flask c., table 2:1.

The pH change caused by respiration when using sea-water without prior equilibration to 10°C (flask b.) represented a 20% increase in hydrogen ion concentration, which was considered to be tolerable, and all future experiments were carried out under these conditions.

2. Levels of radioactivity applied to algae

^{14}C labelled carbohydrate substrates and sodium bicarbonate-^{14}C were supplied at levels of 0.6-2.5μCi/ml. for experimental periods of 1-24 hours. These levels of radioactivity are low by comparison with those used by other workers (Norris et al., 1955; Moses and Calvin, 1959), and it is improbable that cell metabolism has been significantly altered by radiation damage in these experiments.

3. Experimental treatments of algae.

Algae free of epiphytic contaminants were washed in filtered sea-water prior to use. Thalli were used either intact or cut into 1 cm. lengths.

Incubation media consisted of unbuffered, nylon filtered, sea-water containing glucose-^{12}C, glucose-U-, -1-, or -6-^{14}C, or $NaH^{14}CO_3$, or a mixture of these, as indicated in the experimental method.

When CO_2 (or $^{14}CO_2$) released in the dark metabolism of the above compounds was to be collected, a small phial containing 10% KOH was included in each of the conical flasks. These, after addition of the algal tissues, were hermetically sealed.

In heterotrophic experiments the flasks were wrapped in aluminium foil to exclude light. Incubations were conducted at 10°C in a Gallenkamp incubating bath, shaking at 90 oscillations per minute.

Experimental determinations of photosynthesis and photoassimilation were conducted under the conditions of light described in section 7 of the analytical procedures which follow.

At the end of the incubation period the tissues were subjected to one or more of the following assay techniques.

Analytical procedures

1. Extraction of soluble sugars, sugar alcohols, and sugar phosphates.

At the end of experiments, algae were quickly rinsed in 2 ml. of sea-water; this, and the residual incubation medium together constituted the 'final medium'. Algae were then immersed in ice-cold sea-water and washed in a metabolic shaker-bath in darkness at 1°C for 1 hour. This washing time was a compromise between the complete elution of non-metabolised exogenous sugar contained in the cell free-spaces and its heterotrophic uptake beyond the experimental incubation period. After 1 hour the algae were removed from the 'washing medium' and rapidly killed in near-boiling 80% ethanol.

Extraction was completed by three changes, each of approximately 10ml. of hot 80% ethanol, allowing at least 2 hours between changes. These extracts collectively constituted the 'ethanol soluble fraction'. This fraction was then either used directly for analysis or evaporated to dryness at 35°C and made up to constant volume in 80% ethanol. The dry matter present in the alcohol soluble extract represented less than 10% of the total dry weight of tissue in Fucus spiralis and Pelvetia canaliculata.

That fraction which remained after alcohol extraction

will be referred to as the 'alcohol insoluble fraction'. It was used only for radioactive assay and was not analysed further.

2. Colorimetric methods for the quantitation of glucose

(a) Somogyi technique

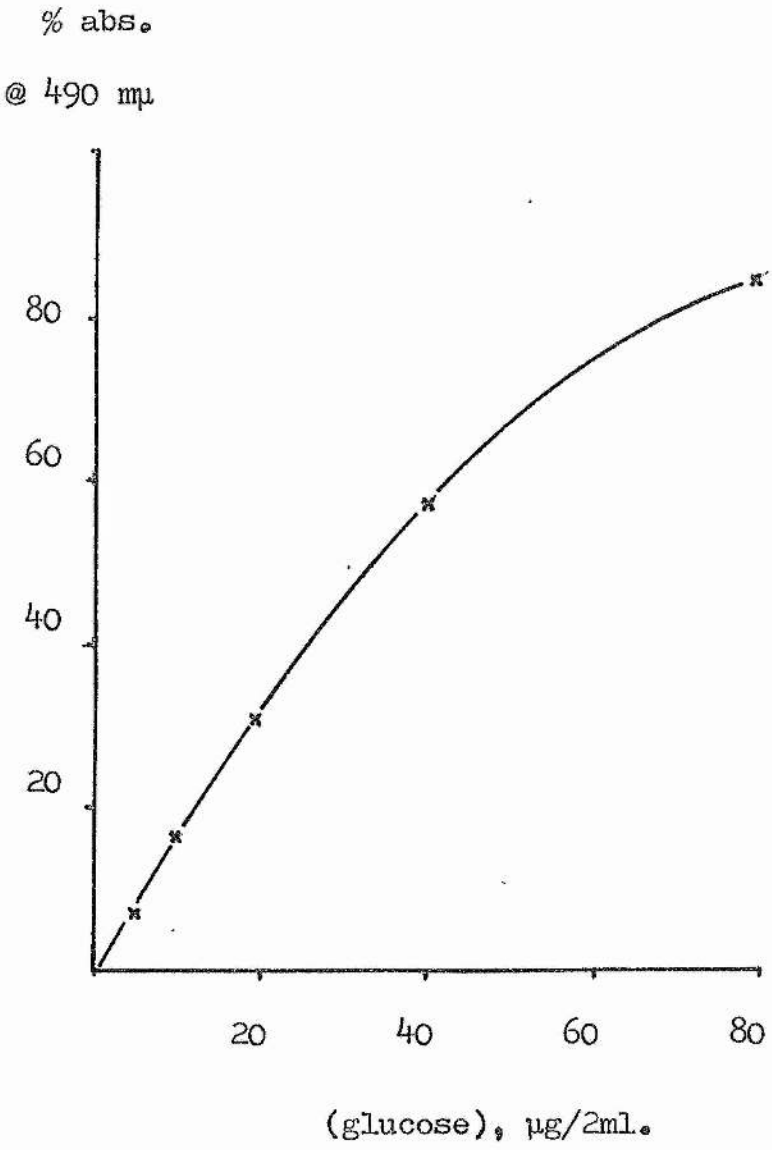
This technique was used as described by Somogyi (1952) in conjunction with Nelson's arsenomolybdate chromogenic reagent (Nelson, 1944). De-proteinisation of the sea-water media analysed was not necessary. % transmission of the coloured solution obtained was determined at 520nm in 1cm. path glass cells, using a Pye Unicam SP 600 spectrophotometer.

In sea-water media the technique was found to be insensitive to glucose concentrations indicated by Somogyi (600 - 100µg glucose/sample). At higher sugar concentrations the chromogenic reagent, whilst functional, was extremely unstable, probably due to the buffer action of the sea-water. Results obtained from this method were so variable that it was abandoned in favour of the Dubois method described below.

(b) Dubois' method

The phenol-concentrated sulphuric acid method of Dubois (1956) yielded reproducible results in the required range of glucose concentration in sea-water, and the colour of solutions remained stable for several hours at 25°C.

Figure 2:2. Calibration curve for the colorimetric estimation of glucose in sea-water by the method of Dubois (1956).



Procedure

(i) to 2ml glucose/sea-water medium, add 1 ml. phenol (5% in water) and 5 ml. concentrated H_2SO_4 (98% analar).

(ii) shake for 15 minutes at 25°C, then read % transmission at 490nm.

3. Respirometry

Rates of respiration were measured at constant temperature in darkness by following manometrically the uptake of oxygen per unit time, using the "direct" method of Warburg (in Umbreit et al., 1957). Oxygen uptake was determined in the presence of 0.5ml. 10% KOH; the possible effect of zero partial pressure of CO_2 on decarboxylation reactions has been found to be negligible (Gibbs, 1962).

Results are expressed as Q_{O_2} values, in micro-litres O_2 consumed per mg. dry weight of alga per hour.

In many experiments respiration was followed not manometrically, but by the estimation of $^{14}CO_2$ released during the metabolism of exogenously supplied ^{14}C-glucose. Algae were incubated in 25 or 50ml. hermetically sealed conical flasks in darkness. $^{14}CO_2$ was trapped in 10% KOH contained in small phials within the flasks. At the end of the experiments the KOH was added to excess saturated $BaCl_2$, containing 5% ammonium chloride, in a centrifuge tube. The

$Ba^{14}CO_3$ precipitate was centrifuged down, washed twice in hot distilled water with re-centrifugation, and then suspended in a constant volume of distilled water. In earlier experiments this suspension was filtered, but as self absorption losses on the filter were high, this method was abandoned in favour of direct radio-assay of aliquots of the precipitate dried on aluminium planchets. As $^{14}CO_3^=$ is known to exchange with atmospheric CO_2, samples were counted immediately after drying.

4. Radioactive assay

Estimations were made of radioactivity in final media, washing media, ethanol soluble and insoluble fractions, $^{14}CO_2$ released in respiration, and in metabolites isolated on paper chromatograms.

Aliquots of liquid media were dried on planchets on a hotplate at 40°C and counted directly.

Ethanol insoluble residues were dried at 110°C for 24 hours, weighed, fragmented, and evenly distributed on lightly greased planchets. Correction for self-absorption by the tissues was made according to data from Drew (1969).

$^{14}CO_2$ released in respiration was counted as $Ba^{14}CO_3$ as discussed earlier (see Respirometry).

Counting equipment

Some samples were counted using a thin end-window

Geiger-Muller tube in conjunction with an Ecko Automatic Scaler (Type N530F). Sample changing was manual. A high intrinsic background, low counting efficiency (1-2%) of ^{14}C β-emissions, and a considerable dead-time combined to make this system impractical.

Thereafter, a Nuclear Chicago automated proportional gas-flow system was used, employing 90% argon, 10% methane. Short dead-time (less than 6μ sec.), low intrinsic background (10-17cpm), and efficiency of about 20% for ^{14}C when using 'Micromil' windows, are characteristic of this equipment. Samples on planchets were counted three times for 10 minute periods and background was counted at the beginning and end of each series.

Counting corrections

Corrections were made for intrinsic background, self-absorption, and, where necessary, for detector dead-time.

Background corrections were made by simple subtraction.

Self-absorption corrections were made to "infinite thinness" by use of the curve (figure 2:3) which was calculated from the formula

$$\frac{n_{true}}{n_{observed}} = \frac{1-(\frac{1}{2})^{x/d_{\frac{1}{2}}}}{0.693x/d_{\frac{1}{2}}}$$

x = sample thickness (mg/cm^2)

* $d_{\frac{1}{2}}$ = half thickness (mg/cm^2)

Dead-time corrections were made as follows. If a counter has a dead time, t, then the total insensitive time per unit time = Rt, where R is the observed counting rate.

If R' is the number that would be recorded if there were no dead-time (no coincidence losses) then the number of lost counts per unit time

$$= R' - R$$

and is given by the product of R' and the fraction of insensitive time Rt.

hence

$$R' - R = R'Rt$$

or $$R' = \frac{R}{1 - Rt}$$

Dead-time correction was applied to samples exceeding 7000 cpm. At 7000 cpm. 96% of counts are recorded assuming 6μ sec. dead-time as quoted by the manufacturers of the equipment.

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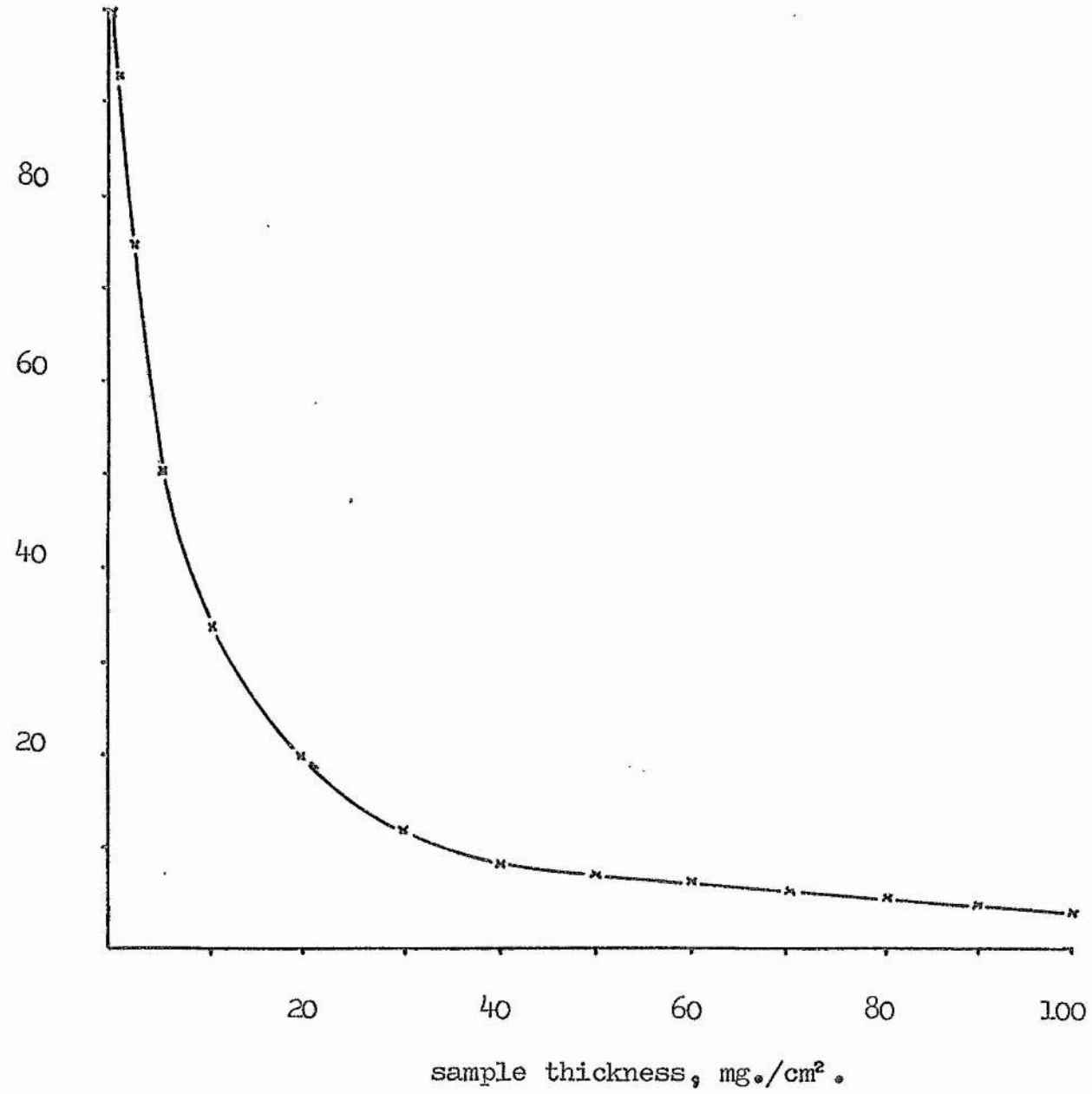
* The "half thickness" is the "thickness" in mg/cm^2 which will stop half the β particles of maximum energy E (0.159MeV for ^{14}C) and is about 0.1 of the range.

$$d_{\frac{1}{2}} = 32E_{max}^{1.33} \text{ mg/cm}^2$$

Figure 2:3. Correction curve for self- absorption losses in counting carbon-14 samples.



Expression of results

Unless otherwise specified, results are expressed as counts per minute (cpm) per mg. dry weight of ethanol extracted alga. Specific radioactivities of respired $^{14}CO_2$ are expressed as cpm/mg. dry wt./µg. C respired; the specific activities of isotopes as supplied by the Radiochemical Centre, Amersham, are given as mCi/M.

5. Paper chromatography

Descending paper partition chromatography was used for the separation of carbohydrates in alcohol soluble fractions of intact algae and particulate preparations.

Alcoholic extracts were taken to dryness in a rotary film evaporator and made up to constant volume in 80% ethanol. Samples were applied to the baseline of chromatograms using Drummond 50µl Microcaps. Spots were rapidly dried in a cold air stream and were limited to 5mm. diameter.

Whatman chromatography paper No. 1 was used exclusively, and chromatograms were run at room temperature in Shandon all-glass two-dimensional tanks.

When it was desired to re-chromatograph any individual compound the region of the spot was cut from the developed chromatogram and eluted by irrigation with water (see Smith, 1960). Tests using radioactive glucose indicated that recovery in the first three droplets exceeded 95%. Carbohydrate-borate

complexes produces by development in the borate solvent system (see below) were less easily eluted until treated with 4% hydrofluoric acid in acetone, after the method of Britten (1959); this treatment was also necessary prior to detection of polyols by ethanolic silver nitrate.

Solvent systems

Chromatographs were developed with one of the following solvent systems. In each case an hour was allowed for equilibration.

Tertiary butanol, picric acid, water

2.2gm wet picric acid was dissolved in 100 ml. 80% t-butanol (Hanes and Isherwood, 1949). This system separates mixtures into various fractions (organic phosphates, carbohydrates, organic acids etc.) with the solvent front still on the paper.

Ethyl methyl ketone, glacial acetic acid, saturated boric acid (9:1:1)

This solvent is that of Rees and Reynolds (1958). It has been used by Britten (1962), Ballio, Di Vittorio and Russi (1964), and Lewis and Smith (1967). It has the advantage of separating polyols from their corresponding sugars , and also gives highly satisfactory results without de-proteinization or de-ionization.

Iso-propanol, n-butanol, water (14:2:4)

This solvent was used due to its relative insensitivity

to temperature (Smith, 1960); it does, however, have the disadvantage that glucose and mannitol, two important metabolites in the present study, run very close together.

Location of Compounds

The distribution of compounds on developed chromatograms was determined chemically and/or by radioassay.

Chemical location

Alkaline silver nitrate

This was used for the detection of carbohydrates after the method of Trevelyan, Proctor and Harrison (1950), and produced grey to dark brown spots on a pale background. These were made permanent, and the background removed, by dipping in 0.880 ammonia and washing in running tap-water for 30 minutes.

Ninhydrin

This reagent was used for the detection of amino acids; it was prepared as 0.2% indane-trione hydrate in acetone (w/v) after Smith (1960), or 0.1% in ethanol with 5% collidine (Levy and Chung, 1953). The reagent was sprayed using a Shandon Laboratory Spray Gun, and mauve spots developed after 2-3 minutes heating at 110°C in a drying cabinet.

Detection of radioactivity on chromatograms

Strips of chromatography paper 4cm. wide corresponding to single initial baseline spots were scanned for radioactivity

using one of two methods.

(a) A thin-end window Geiger-Muller tube with a 2mm. wide collimator slit width was moved manually along the paper at 5mm. intervals. Radioactivity was recorded using a Panax Model 5054 Ratemeter. Employing the maximal integrating time of 25 secs, scanning was time consuming, and as sensitivity was low the high sensitivity, automated method described below was used as soon as the equipment was available.

(b) A Nuclear Chicago Actigraph 111 4 Strip Scanner was used for subsequent detection of radioactivity on paper chromatograms. The recorder incorporated a disc integrator and thus quantitative data could be obtained for the distribution of radioactivity. This was expressed as % of total activity on the chromatogram, and thus the extent to which a reaction had proceeded was known.

This technique was particularly useful when used in conjunction with GLC analysis. Quantitation of peaks obtained by the latter method and knowledge of radioactivity within these peaks, were together used in the determination of specific activities of metabolites, thus giving information about metabolic pool sizes and turnover rates during experiments.

Identification of compounds

Authentic marker spots were run simultaneously with all

chromatograms; such spots included glucose-U-^{14}C and mannitol-1-^{14}C as markers for radioactive scanning.

For monosaccharides and polyols, mobilities identical with a known marker in three solvent systems were taken as indication of chemical identity, and for radioactive compounds this was usually further substantiated by elution and co-chromatography with the labelled or unlabelled authentic compound.

Acid hydrolysis

Suspected disaccharides were eluted from chromatograms and 0.2ml. of eluate hydrolyzed with 5ml. 5N-HCl at 110°C for three hours. The acid was removed by rotary evaporation. 0.1ml. distilled water was then added and chromatographic mobilities of the possible new compounds were compared with appropriate authentic marker compounds. Identical mobility of the disaccharide with authentic marker and hydrolysis of the disaccharide to its correct constituent monosaccharide(s) was taken as evidence of chemical identity.

Treatment of phosphates with phosphatase

Suspected phosphates were treated with human seminal acid phosphatase after the method of Smith (1960).

The spot was eluted from the paper with water and concentrated by rotary evaporation to 0.25ml. Then, 0.025ml. of 0.2M acetate buffer (pH 5) containing 0.01M-$MgCl_2$ was

added. 10µl. of the supernatant liquid from centrifuged semen were added and the mixture incubated for $3\frac{1}{2}$ hours at 37°C. Simultaneous treatment of an authentic sugar phosphate was carried out to verify phosphatase activity, and a third incubation in ethanolic acetate buffer was used as a control. Identity of resultant monosaccharides was subject to the same conditions as outlined earlier.

Enzymic hydrolysis of putative trehalose

A crude trehalase preparation was extracted from the South American cockroach Blaberus discoidalis after the method of Kalf and Reider (1958). The preparation was assayed using authentic trehalose substrate; paper chromatography showed glucose to be the sole product.

6. Gas-liquid chromatography

A Pye Automatic Preparative Chromatograph Series 105 Model 15 was used in this study for the quantitative analysis of sugars and sugar alcohols from brown algae.

Glass columns, 5' x $\frac{1}{4}$" (internal diameter) with glass-metal sealed outlet were used.

Coating of support material

Acid washed, siliconised Diatomite 'C' (60-72 mesh) was used as the support material, and 2% SE 52 (phenyl silicone gum) as the liquid stationary phase.

The appropriate quantity of stationary phase was

dissolved under reflux in dichloromethane (DCM) and added to the siliconised Diatomite 'C' in a large evaporating flask. DCM was then slowly removed by continuous rotary evaporation under moderate vacuum until the coated Diatomite was dry.

Column packing

Columns were packed by application of moderate vacuum to the outlet. In this way packing was continuous and even, and was achieved rapidly. Finally, with the column in position in the GLC oven, 10psi of carrier gas (nitrogen) was applied and the column pre-heated at 250°C for 12 hours.

Volatile derivatives

Volatile trimethylsilyl (TMS) ethers of sugars and sugar alcohols were prepared after the method of Sweeley et al., 1963. Alcoholic extracts of algae were evaporated to dryness and re-dissolved in 1ml. of anhydrous pyridine. 0.2ml. of hexamethyldisilazane (HMDS) and 0.1ml. of trimethylchlorosilane (TMCS) were added, and the mixture allowed to stand overnight in a stoppered flask at room temperature before analysis.

Method of analysis

10µl aliquots of silylated carbohydrate mixtures were injected onto the column at 140°C. The temperature

programme was adjusted so that clear separation of components was achieved in less than 20 minutes (i.e. 8°C/min. rise to a maximum of 250°C).

Identification of peaks

Identification of compounds was based on their retention times under given conditions. Co-chromatography with authentic sugar or sugar alcohol was considered as evidence of chemical identity. An example of sugar and polyol separation is shown in figure 2:4. Here, a temperature rise of 4°C/min. was used to ensure good separation.

Quantitative estimation of peaks

The linear response of flame ionisation detection for TMS derivatives of carbohydrates (but c.f. Cayle et al., 1968) allows for accurate quantitation from GLC pen recorder scans. This was carried out by a disc integrator on the recorder itself. Integrated peak areas were compared directly to those obtained for known amounts of the authentic compounds, and quantitation was by simple mathematical proportion.

7. Light intensities

In photosynthesis and photoassimilation experiments an Isco Spectroradiometer was used to determine incident light energy in the range 380-750nm. Light measurements were made via a 6' remote probe situated beneath a conical incubation flask in a Gallenkamp metabolic shaker. Correction

factors for light loss were applied, and scans were integrated mathematically.

Experiments were conducted in conditions of both high and low light intensity. In high light intensity experiments 2 x 1500 Watts tungsten-halogen strip lights were fitted above a glass-bottomed water-flow coolant tank, itself situated above the metabolic shaker. Although more spectrally balanced than ordinary tungsten light, the energies of these lights were still somewhat biased towards red light (fig. 2:5a). Low light intensity experiments were made in very dull natural daylight from a North facing window. Energy distribution within the spectrum was here more evenly balanced (fig. 2:5b).

Incident light energy is expressed as calories per minute per cm^2.

Figure 2:4. GLC trace of the TMS derivatives of a mixture of polyols and sugars on a 5' glass column packed with 2% SE 52 on Diatomite 'C' (60-72 mesh).

a = dichloromethane solvent peak

b = glycerol

c = arabitol

d = fructose

e = α galactose

f = α glucose

g = β galactose

h = mannitol

i = β glucose

j = sucrose

k = trehalose

N_2 carrier flow-rate 40 ml/min. at 5 psi.

Figure 2:4.

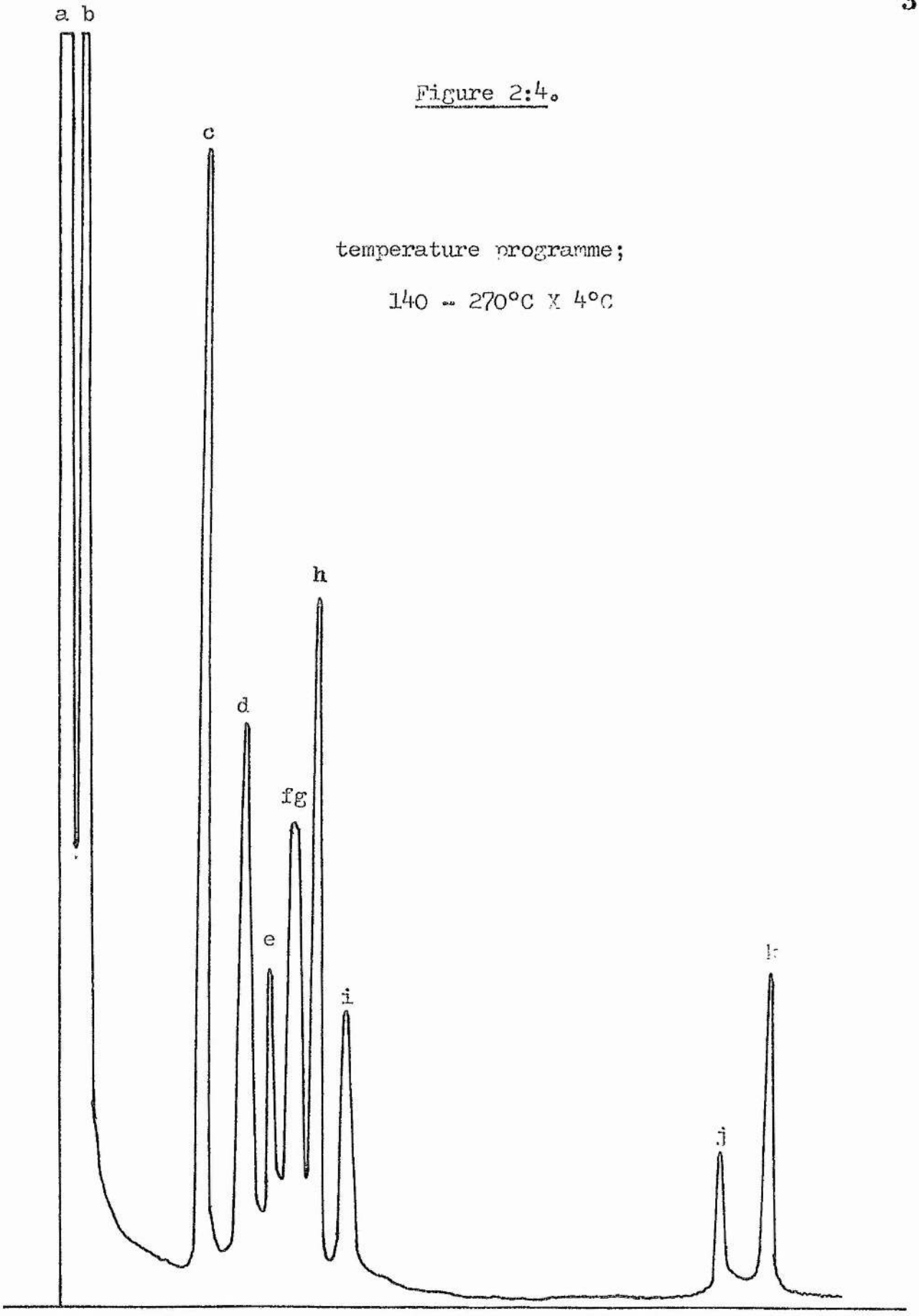
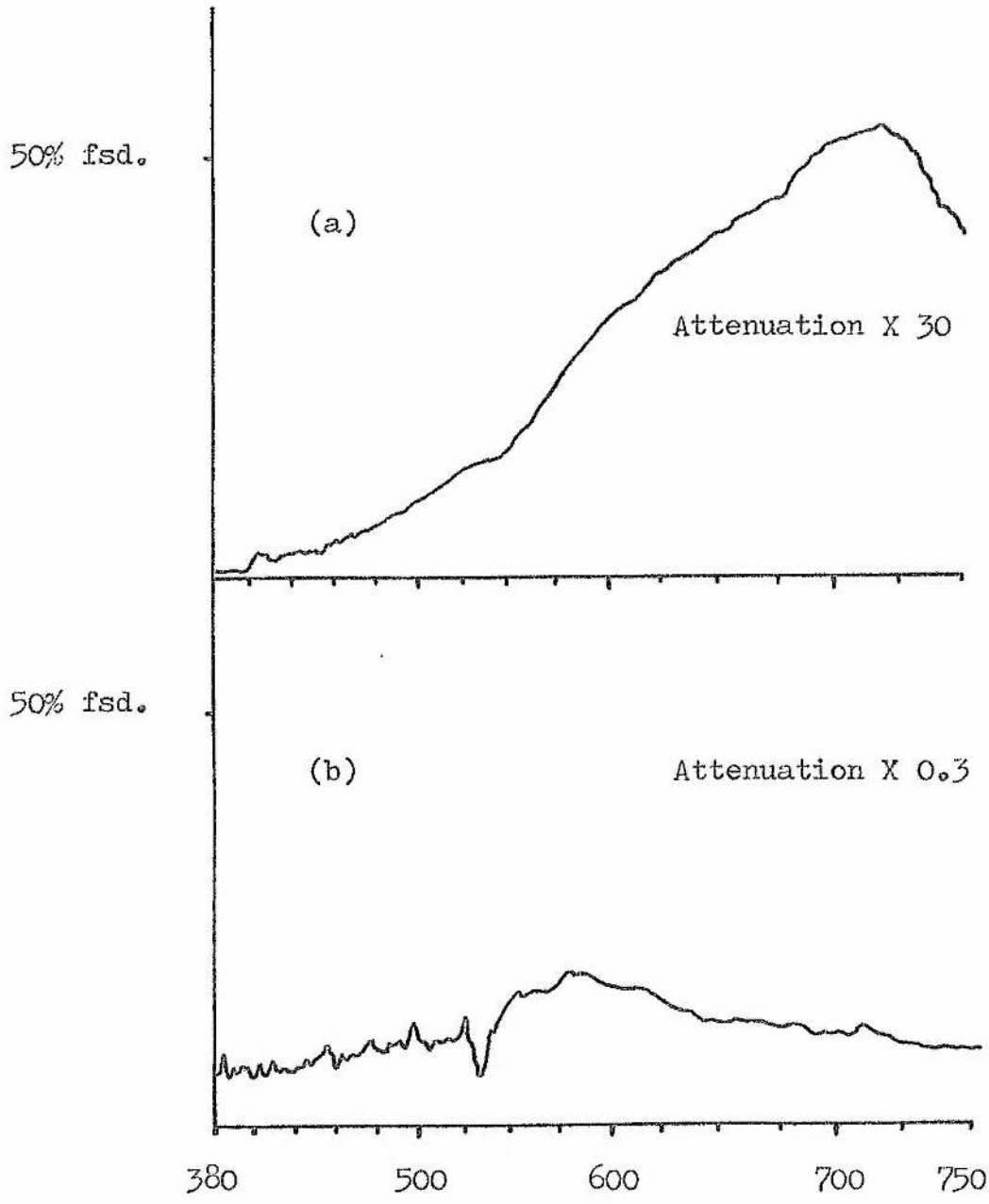


Figure 2:5.

Spectroradiometric analysis of incident light energy.

(a) high light intensity supplied by tungsten-halogen.

(b) low daylight intensity.



(a) 0.245 cal/min/cm^2.

(b) 0.00145 cal/min/cm^2.

RESULTS

CHAPTER 3

Low molecular weight carbohydrates in the Phaeophyceae.

Contents page

Introduction

The occurrence, physiology and biochemistry of low molecular weight carbohydrates in the Phaeophyceae is moderately well documented. Touster and Shaw (1962) have considered metabolic aspects of polyols, and Lewis and Smith (1967) have dealt extensively with the distribution, physiology and metabolism of polyols in plants. It is here proposed to bring together information concerning the Phaeophyceae.

Of the various low molecular weight carbohydrates found in the Phaeophyceae, the acyclic polyols, and particularly mannitol, are quantitatively of the greatest significance. Lewis and Smith (1967) have listed twenty-seven genera in which mannitol has been found, and indeed there is only one report of the definite absence of this polyol in members of Phaeophyceae (Kylin, 1944).

Quantitative estimations reveal that mannitol may contribute a significant proportion of the dry weight of brown algae, and this proportion has repeatedly been demonstrated to be controlled largely be environmental factors, particularly "seasonal change". The results of Black (1950) suggest that for Laminaria hyperborea day length controls mannitol levels; he reports for the Orkneys, an annual minimum of 8% in March and a summer maximum of 35% dry weight, whereas further South, In Cullipool, the same species had a summer maximum of 26%.

In each case the polysaccharide laminarin showed a parallel variation. In South Africa, where seasonal variation is less marked, Holdt et al. (1955) demonstrated winter mannitol maxima and an insignificant laminarin change.

Reproductive status of the plants has also been shown to affect mannitol levels. Moss (1948, 1950) showed that the negative gradient of mannitol content from apex to base in vegetative thalli of Fucus was lost at the onset of reproductive activity. The mannitol content of receptacles diminished with maturity, and Moss (1950) suggested that the mature gametes do not store this polyol.

Bidwell (1958) demonstrated that mannitol was the major soluble product of photosynthesis in seven genera of Phaeophyceae. From data obtained in experiments in which mannitol-1-^{14}C was offered to Fucus vesiculosus, Bidwell and Ghosh (1962) concluded that mannitol was not, however, the respiratory substrate. Explanation of this anomaly in terms of the vast difference between the internal and external specific activities of mannitol has been offered by Lewis and Smith (1967), and in response to work by Yamaguchi et al. (1966) on Eisenia bicyclis, Bidwell (1967) has re-appraised the situation and come to agree with the contemporary opinion that mannitol is the respiratory substrate in these algae.

Mannitol has also been implicated in other roles, such as the maintenance of high internal osmotic pressure (Lewis and Smith, 1967), which is usually somewhat higher than that of sea-water (Guillard, 1962). Also, Bourne (1958) has suggested that polyols might function in the protection of injured tissue.

In tissues other than those of marine algae certain other features of polyol metabolism have been established. Edson (1953) has demonstrated that co-enzymes and polyol dehydrogenases mediate the interconversion of sugar isomers via polyols in male accessory glands. This system has also been described in fungi, but not in the algae.

Since polyols are more highly reduced than the corresponding sugars they are eminently suitable as storage compounds. It has been proposed by Touster and Shaw (1962) that where polyols are not serving specifically in metabolism they might function in the transfer of hydrogen on co-enzymes, and thereby in the control of the various metabolic pathways.

In addition to mannitol two other polyols are known to occur in the Phaeophyceae. One of these, volemitol, was found by Lindberg and Paju (1954) in Pelvetia canaliculata, the only brown alga known to contain a heptitol. The other, laminitol, is a cyclic polyol (cyclitol), and has been found in L. hyperborea (Lindberg and McPherson, 1954), F. spiralis

and Desmarestia aculeata (Bouveng and Lindberg, 1954). Laminitol is present only in trace amounts, and like volemitol it has no known metabolic role.

Besides the free polyols, Lindberg and his colleagues have isolated and identified polyol glucosides and acetates from various brown algae, and Lindberg and McPherson (1954) have made positive identification of the free reducing sugars laminarobiose and laminarotriose in L. hyperborea. These might have been present as the biosynthetic precursors of laminaran or products of its autolytic degradation. The presence of glucose and fructose is attributed to the hydrolysis of sucrose, and trehalose has been tentatively identified in F. vesiculosus by Lindberg (1953). These data are summarised in table 3:1.

Key to table 3:1.

+	positive identification
-	absent
T	trace amount present

1. Lindberg and McPherson (1954)

2. Lindberg (1953)

3. & 5. Bouveng and Lindberg (1954)

4. Lindberg and Paju (1954)

Table 3:1. The distribution of low molecular weight carbohydrates in brown algae.

(summary of data from Lindberg et al., 1953-1954)

	1. Laminaria hyperborea	2. Fucus vesiculosus	3. Fucus spiralis	4. Pelvetia canaliculata	5. Desmarestia aculeata
Mannitol	+	+	+	+	+
Volemitol	-	-	-	+	-
Laminitol	+	+?	+	+?	+
Mannitol monoglucoside	+	+	+	+	+
Mannitol diglucoside	+	+	+	+	+
Volemitol monoglucoside	-	-	-	+	-
Volemitol diglucoside	-	-	-	+	-
Mannitol monoacetate	+	+	+	-	-
Sucrose	T	T	-	T	-
Glucose	-	T	-	T	-
Fructose	-	T	-	T	-
Trehalose	-	T?	-	-	-
Laminarobiose	+	-	-	-	-
Laminarotriose	T	-	-	-	-

Experimental

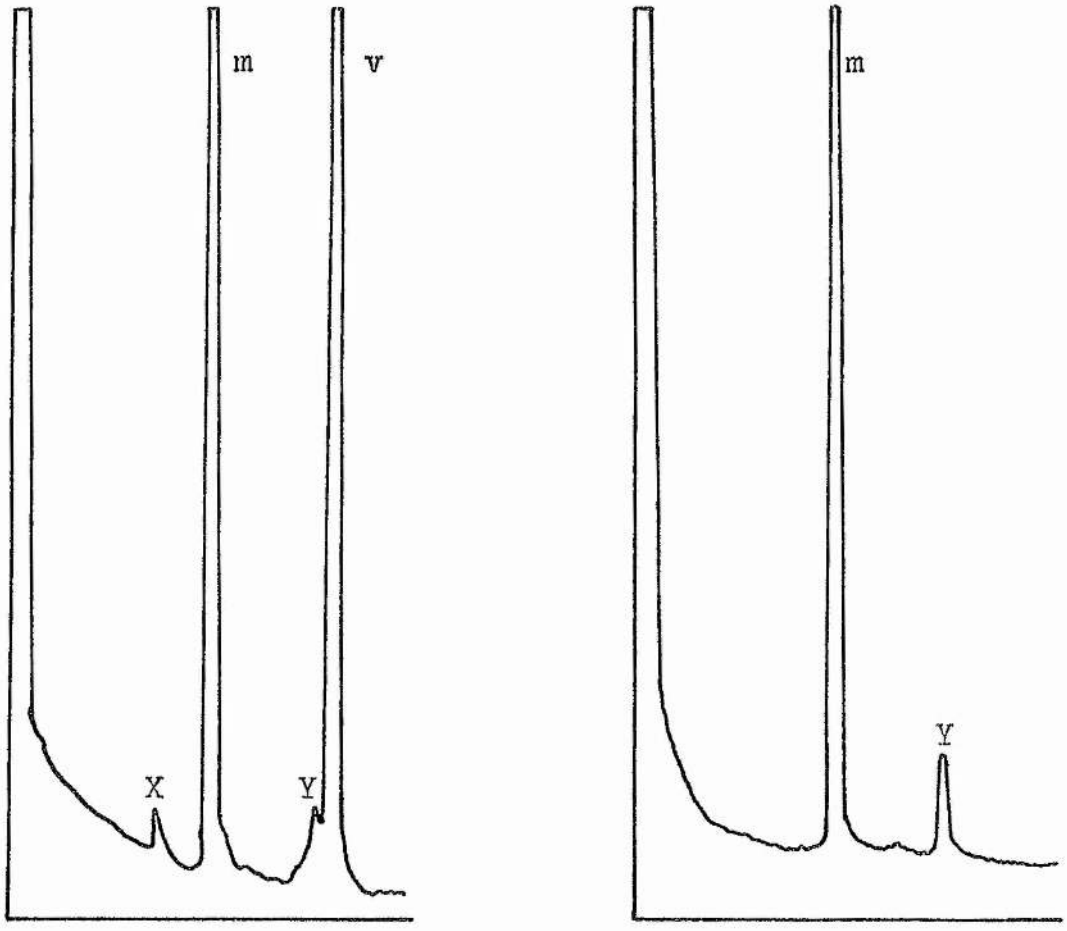
1. Analysis of soluble carbohydrates.

Qualitative analysis of soluble carbohydrates in Pelvetia and Fucus spiralis.

Analysis of ethanolic extracts of both these species by paper and gas-liquid chromatography failed to reveal any free sugars. However, the polyol mannitol was detected by both methods in these species, and also a second polyol, volemitol, in Pelvetia. These results are consistent with those of Lindberg and Paju (1954), and Bouveng and Lindberg (1955), working with the same species. The significance of the virtual ubiquity of mannitol in the Phaeophyta has already been mentioned, and will be discussed at greater length later.

Trace amounts of other carbohydrates have been detected by GLC analysis of ethanol soluble fractions, but only from material collected during the winter. One of these (X in figure 3:1a) was found only from Pelvetia apices, and has a retention time similar to, but not identical with, fructose. The other (Y in figure 3:1b) was found in extracts of Pelvetia, basal tissue of F. spiralis, and in greater amounts in F. serratus. This compound, which had a retention time intermediate between those of α-and β-volemitol, does not correspond to the mono- or di-glucosides found in Pelvetia and F. spiralis by Lindberg and Paju (1954) and Bouveng and Lindberg (1955).

Figure 3:1. GLC traces of the TMS derivatives of ethanol soluble carbohydrates from Pelvetia and F. spiralis.



(a) Pelvetia

X = ($R.T._m$ 0.76)

m = mannitol

Y = ($R.T._m$ 1.52)

v = volemitol

(b) F. spiralis

m = mannitol

Y = ($R.T._m$ 1.52)

$R.T._m$ = retention time relative to that for mannitol

$R.T._m$ for fructose = 0.74 (c.f. X above)

Whether the compound corresponds to the mannitol acetate or the laminitol of the latter authors is not known.

Quantitative analysis of sugar alcohols.

GLC analysis of Pelvetia and F. spiralis yielded quantitative data indicating considerable fluctuation in mannitol and volemitol content with season. Seasonal variation in mannitol content has been reviewed by Boney (1965), and is discussed by Lewis and Smith (1967). Table 3:2 shows values for mannitol content of freshly collected algae in June and January.

Data indicate that for apical tissues of both species maximal mannitol content is reached in summer, which is in general accord with the results reviewed by Boney (1965). However, winter maxima were demonstrated in the basal regions, a situation previously observed only in some South African seaweeds (Holdt et al., 1955). The possible significance of these data will be discussed later in conjunction with the results of translocation experiments.

In Pelvetia, approximately equal amounts of mannitol and volemitol were found, such that the total polyol contents of Pelvetia and F. spiralis are not dissimilar. In both species apical maxima for mannitol were found in summer and basal maxima in winter.

Table 3:2. % Polyol contents of apical and basal tissues of Pelvetia and F. spiralis.

		Pelvetia		F. spiralis	
		Apex	Base	Apex	Base
		(mg. per 100 mg. extracted dry wt)			
Mannitol	June	9.6	3.7	20.6	8.6
	January	7.2	4.5	14.4	18.6
Volemitol	June	11.1	4.9	-	-
	January	6.3	4.6	-	-
Total	June	20.7	8.6	20.6	8.6
	January	13.5	9.1	14.4	18.6

The distribution of mannitol and volemitol along the thallus of Pelvetia.

Data shown in table 3:2 do not imply a gradual decrease in polyol content from apex to base during any given season.

Freshly collected thalli of Pelvetia, each carefully selected for being 6 cm. in length from apex to base, were cut into 1 cm. lengths and analysed for polyols. The thalli used in this experiment were collected in December. The results are shown in figure 3:2 and indicate that maximal polyol content is to be found in the sub-apical tissues.

2. Respiration, and the utilization of endogenous mannitol.

Determination of endogenous respiratory rate in Pelvetia and F. spiralis.

Studies of aerobic respiration have been concerned with oxygen consumption, carbon dioxide release, or both. Because of inherent inaccuracies in the estimation of carbon dioxide released in the respiration of unlabelled metabolites, manometrically determined oxygen has here been considered exclusively. Carbon losses are based on the respiratory quotient (R.Q.) for mannitol, assuming this compound to be the respiratory substrate; justification of this assumption will be presented shortly.

Figure 3:3 shows oxygen consumption by apical and basal tissues of Pelvetia and F. spiralis. Uptake of oxygen (Q_{O_2} values)

Figure 3:2. The distribution of mannitol and volemitol along the thallus of Pelvetia.

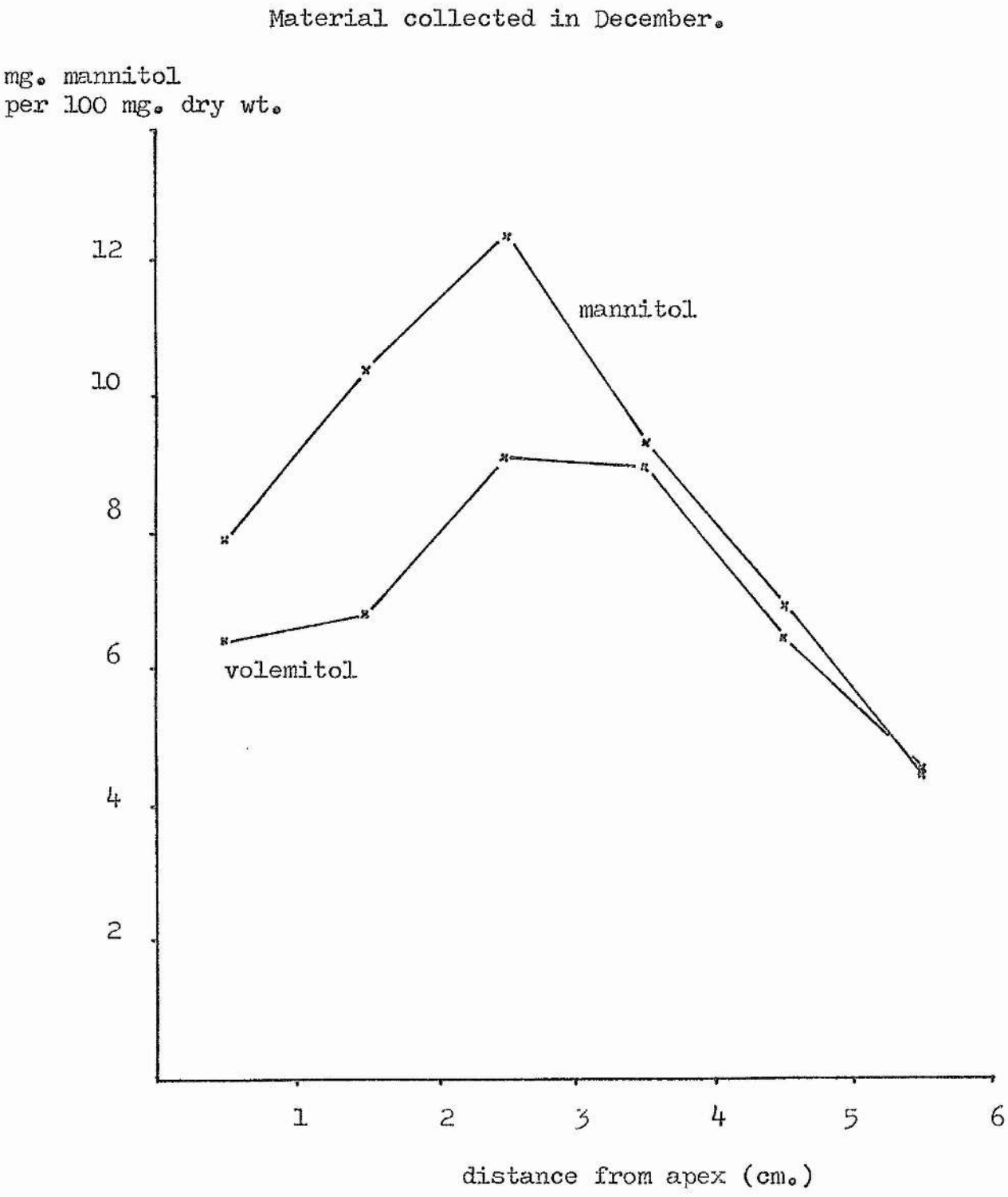


Figure 3:3.

F. spiralis and Pelvetia

(a) 1 cm. lengths of apical tissue

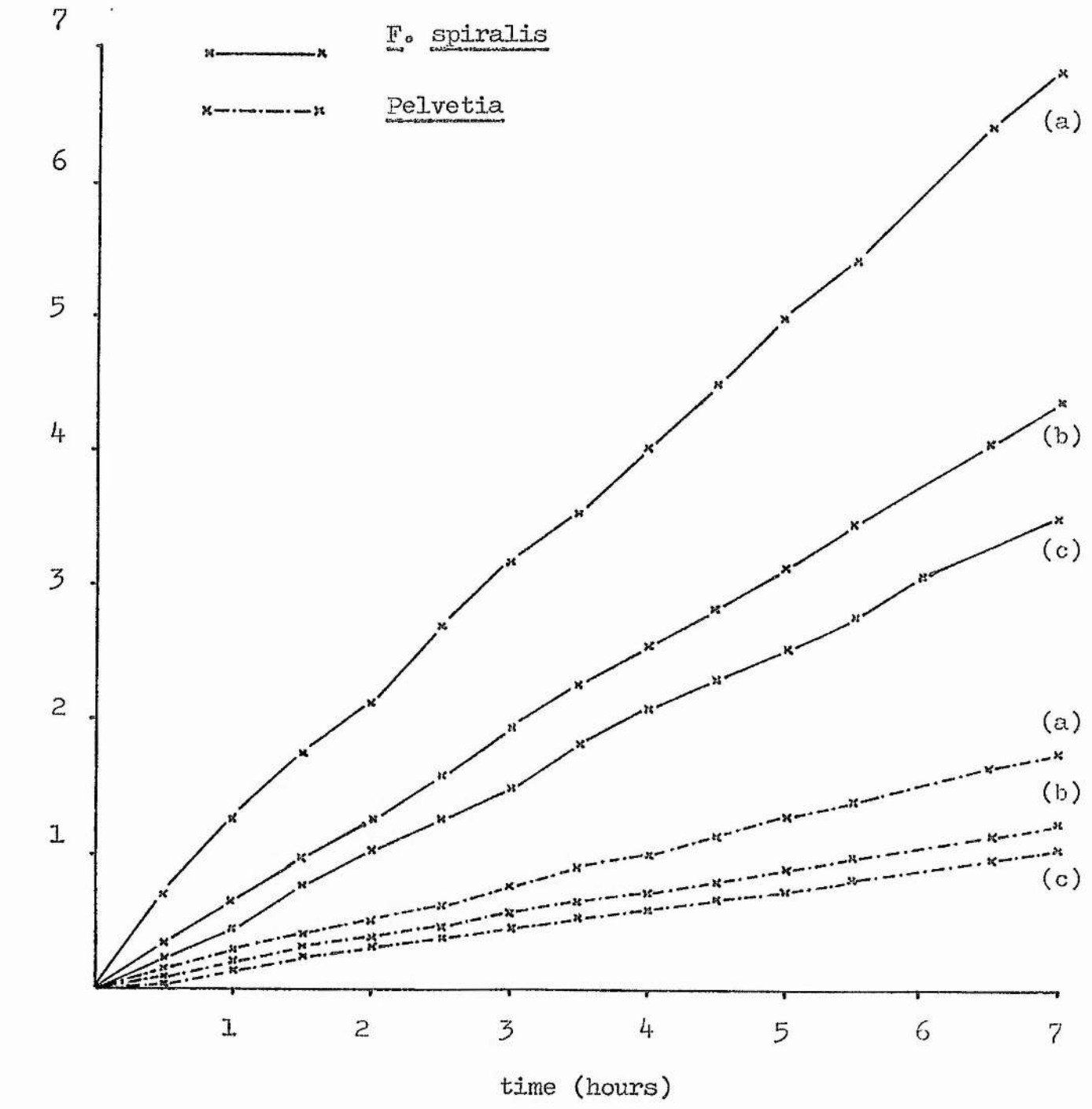
(b) Entire thalli cut into 1 cm. lengths

(c) 1 cm. lengths of basal tissue.

QO_2 values (µl/mg. dry wt./hour)

	F. spiralis	Pelvetia
(a)	0.85	0.25
(b)	0.55	0.18
(c)	0.50	0.15

Figure 3:3. Oxygen consumption by respiring tissues of Pelvetia and F. spiralis.



and calculated carbon losses are shown in table 3:3.

Carbon losses of this magnitude are extremely significant for intertidal plants in which photosynthesis can proceed at maximal efficiency for only a few hours each day, or not at all for several days in the case of Pelvetia during neap tides (Bidwell and Craigie, 1962; but c.f. Sieburth, 1969b, who has demonstrated photosynthesis in emersed intertidal algae moistened by rain-water). In this context the lower carbon loss suffered by Pelvetia might be of considerable ecological significance. Sustained periods of emersion would still incur large losses of carbon, however, and it can be calculated from data in tables 3:2 and 3:3 that the endogenous reserves of mannitol would last for 8-11 days of emersion (calculated as a ratio of total mannitol carbon over carbon loss per day). However, this is assuming emersed and immersed respiratory rates are equal.

Respiratory carbon loss by Pelvetia during emersion.

Manometric evidence suggests reduced respiratory carbon losses during emersion. Dry apical tissue of Pelvetia had a Q_{O_2} of 0.04µl/mg/hr. at 10°C, representing a carbon loss of 0.02µg/mg/hr., which is less than 15% of the loss sustained by the same tissue when immersed (see table 3:3). A similar, if less dramatic, result was obtained by Bidwell and Craigie (1962) in their studies on respiration and photosynthesis in F. vesiculosus.

Table 3:3. Oxygen consumption and calculated carbon losses by *Pelvetia* and *F. spiralis*.

	QO_2 (µl/mg/hr.)	C loss (µg/mg/hr.)
F. spiralis apex	0.85	0.46
F. spiralis base	0.50	0.27
Pelvetia apex	0.25	0.14
Pelvetia base	0.15	0.08

Starvation of emersed Pelvetia in the dark.

Entire thalli of Pelvetia were blotted dry and starved in the dark at 5°C for 14 days. The thalli were then divided into 2 aliquots and treated as follows:

(a) 1cm. lengths of apical tissue were excised and carbohydrate was extracted in ethanol. The mannitol content was determined by GLC analysis, and is designated 'Starved' in table 3:4.

(b) Dry tissue was placed in running sea-water in darkness for 24 hours at room temperature, and then treated as (a) above. This treatment is designated 'Starved + H_2O' in table 3:4.

The actual carbon loss shown in table 3:4 for the 'Starved + H_2O' treatment very closely approximates the manometrically determined value (0.14µg/mg/hr.) for immersed tissue in table 3:3. Similarly, the value for 'Starved' dry tissue is probably not significantly different from that during emersion. The slight discrepancies possibly being attributable to temperature. It thus appears likely that mannitol is indeed the respiratory substrate, at least in Pelvetia, and conceivably in Fucus too. These results are also interesting from the ecological standpoint, in that reduced carbon loss during emersion confers the ability to withstand longer periods of emersion than would otherwise be possible, especially in conditions of high ambient temperature. Another feature of

Table 3:4. Mannitol content of starved apical tissues of Pelvetia. The control represents average mannitol content of fresh material collected at the beginning and end of the experiment.

	Mannitol (mg./100mg. dry wt.)	Volemitol (mg./100mg. dry wt.)	Total C loss (µg)	C loss (µg/mg/hr.)
Control	9.6	11.1	-	-
Starved	8.7	11.0	355	.01
Starved + H_2O	2.6	11.0	3950	.11

possible significance is that a similar depletion of endogenous volemitol was not evident, reserves of this compound remaining virtually constant throughout the above starvation experiment. Thus, it appears that at least during starvation volemitol and mannitol are not interconvertable. Hence, upon re-immersion, the alga has not only an adequate internal O.P., conferred at least in part by volemitol, but also a source of greater potential reducing energy per molecule than mannitol, a factor which may be important in the recovery of metabolic status.

3. The possible role of mannitol in the maintenance of high internal O.P.

The subject of salt balance and cellular O.P. in algae has been reviewed by Guillard (1962). Algae tend to maintain an internal concentration somewhat above that of the medium, thereby conferring turgor upon the cells. In marine algae this O.P. excess may be small, as in Halicystis, or large, as in Valonia, in which the O.P. is approximately twice that of the sea-water medium.

The latitude of osmotic stress tolerated depends ultimately on the ability of the protoplasm to function when its salt concentration is altered. This problem is obviously of particular importance in intertidal algae, which are subject to extreme variations in environmental conditions.

Lewis and Smith (1967) have postulated on theoretical

grounds that metabolically inactive components of polyol pools might serve as endogenous osmo-regulators. Black, however, (1950) reports that when Laminaria saccharina is put into distilled water both salts and mannitol diffuse rapidly out of the tissues. Sieburth (1969b) has shown that organic carbon exudation induced by natural rainfall ranges from 7.5 mg/100g./mm. rain in Chondrus to 46.4 mg. in F. vesiculosus.

From the available data it is possible to make at least approximate calculations to test the hypothesis of Lewis and Smith.

$O.P. = nRT$ where n = molarity
R = gas constant (0.082 litre atmospheres)
T = absolute temperature

When the salinity of sea-water is 35‰ the O.P. at 10°C is 23.3 atmospheres (Sverdrup et al., 1949). Substituting in the above formula 'n' can be calculated as ~1 M, equivalent to 182 g. mannitol/l., or 0.5 M for a dissociated electrolyte such as NaCl (29 g. NaCl/l.). These are the concentrations of NaCl or mannitol which would be required to maintain an internal O.P. equal to that of the sea-water medium. No O.P. excess, and hence turgor, has been allowed and hence the following estimates are conservative.

If we consider the cell to be a solution of mannitol,

and that approximately 200mg. of tissue are equivalent to 1 ml., then from table 3:2 it can be seen that in Pelvetia apical tissue in June this represents 9.6 x 2 = 19.2 g. mannitol/l. This confers an O.P. of 19.2/182 x 0.082 x 283 = 2.53 atmospheres; this is about one-ninth that required to maintain isotonicity with the external medium. The heptitol volemitol in similar concentrations will confer a slightly lesser contribution to the O.P. in this species.

Other values calculated from data given in table 3:2 are presented in table 3:5.

From these calculations it is apparent that the maximal O.P. conferred by mannitol (or mannitol plus volemitol) is 5.28 atmospheres, in Fucus apical tissues. This is less than 23% of the required O.P. for isotonicity with the external medium, and is thus probably not a major factor in endogenous osmo-regulation in these species.

Table 3:5. O.P. conferred by mannitol and volemitol to apical and basal tissues of F. spiralis and Pelvetia in June and January.

		June			January		
	polyol	polyol content	O.P.		polyol content	O.P.	
Pelvetia apex	mannitol	9.6	2.53	4.75	7.2	1.84	3.22
	volemitol	11.1	2.22		6.3	1.38	
Pelvetia base	mannitol	3.7	0.95	2.03	4.5	1.25	2.26
	volemitol	4.9	1.08		4.6	1.01	
Fucus apex	mannitol	20.6	5.28		8.6	2.20	
Fucus base	mannitol	14.4	3.69		18.6	4.76	

Note: (1) polyol contents are expressed as mg./100mg. dry wt.

(2) the O.P. of sea-water of salinity 35‰ at 10°C is 23.3 atmospheres.

CHAPTER 4

The Heterotrophic uptake and Metabolism of Exogenous Glucose in the Phaeophyceae.

Contents page

Introduction

The photosynthetic production of mannitol in the Phaeophyceae has been demonstrated by Bidwell (1958), Bidwell et al. (1958) and Yamaguchi et al. (1966). Drew (personal communication) has shown both volemitol and mannitol to be formed during photosynthesis in Pelvetia canaliculata.

The fate of organic carbon accreted heterotrophically is less well documented, and moreover, its quantitative significance may, in the light of recent research by Sieburth (1969b), be extremely dubious. Sieburth showed exudation of organic carbon during photosynthesis in Fucus vesiculosus; although Laminaria digitata and L. agardhii also lost carbohydrate in darkness, F. vesiculosus did not. The Laminarias are not efficient heterotrophs, at least of carbohydrate substrates (Drew, 1969), and should dark exudation take place to the extent indicated by Sieburth, then it is unlikely that heterotrophy could play a significant role in the negation of carbon losses by these algae. The same cannot be said a priori of Fucus. If as Sieburth found, no carbon losses occur in darkness, it is possible that Wilce's (1967) hypothesis might be substantiated; that heterotrophic uptake might indeed offset respiratory carbon losses in the dark.

The findings of Bidwell and Ghosh (1963) suggest considerable glucose uptake by F. vesiculosus with little

subsequent metabolism, but Drew (1969) showed that much of this glucose had entered the "free space" of the algal tissues, hence the lack of further metabolism. From the results of Bidwell and Ghosh (1963) it is evident that some glucose had entered the metabolic pathways, but none of this carbon was found in mannitol. Lin and Hassid (1966) have similarly shown that F. gardneri does not convert exogenously supplied glucose to manntiol.

Drew (1969) showed that for Pelvetia and Ascophyllum nodosum grown in darkness in glucose media, less than 5% of glucose taken up was elutable, and further, that these species were both able to produce mannitol heterotrophically. Also, apical tissues of these species were able to take up far more exogenously supplied glucose than apices of F. vesiculosus.

It has been suggested by Lwoff et al. (1949, 1959) that hexokinase deficiency might account for apparent glucose blocks, by preventing the conversion of free glucose to phosphorylated derivatives which are generally assumed to be the actual metabolic intermediates. Increased respiratory rate in the presence of exogenous carbohydrate has been taken to be indicative of hexokinase activity by Taylor (1950, 1960 a, b) and by Jacobi (1957 a, b). Hexokinase activity has actually been demonstrated in F. gardneri by Lin and Hassid (1966), whilst Bean and Hassid (1956) have demonstrated direct

oxidation of nonphosphorylated sugars to their corresponding acids in the red alga Iridiophycus flaccidus.

Experimental

1. Loss of carbohydrate from immersed F. spiralis in darkness.

In experiments in which exogenous unlabelled glucose was supplied to apices of F. spiralis in darkness, glucose uptake was estimated by disappearance of glucose from the external medium. It soon became apparent that final media contained more, not less, carbohydrate as estimated by the method of Dubois (1956), than did the initial media. Evidently the tissues had lost some carbohydrate during incubation. Simultaneous controls, which initially contained no exogenously supplied carbohydrate, also appeared to contain carbohydrate in the incubation media at the end of the experimental period. The results of such experiments were variable and will not be presented in detail here. Values for leakage from the controls, however, were reproducible, and typical data are shown in figure 4:1a. Incubation media were 2ml. sea-water, and (by extrapolation of the log data) after 1.75 hours equilibrium was attained, when total carbohydrate loss was about 150µg/100 mg. dry wt. This unexpected leakage was attributed to disruption of cells when apices were excised with consequent release of soluble carbohydrate, probably mannitol.

Similar experiments using intact thalli (entire sporelings) of F. spiralis also showed leakage of carbohydrate, albeit less than that from the excised apices (figure 4:1b). In both cases

Figure 4:1a. Carbohydrate leakage from excised apical tissue of F. spiralis.

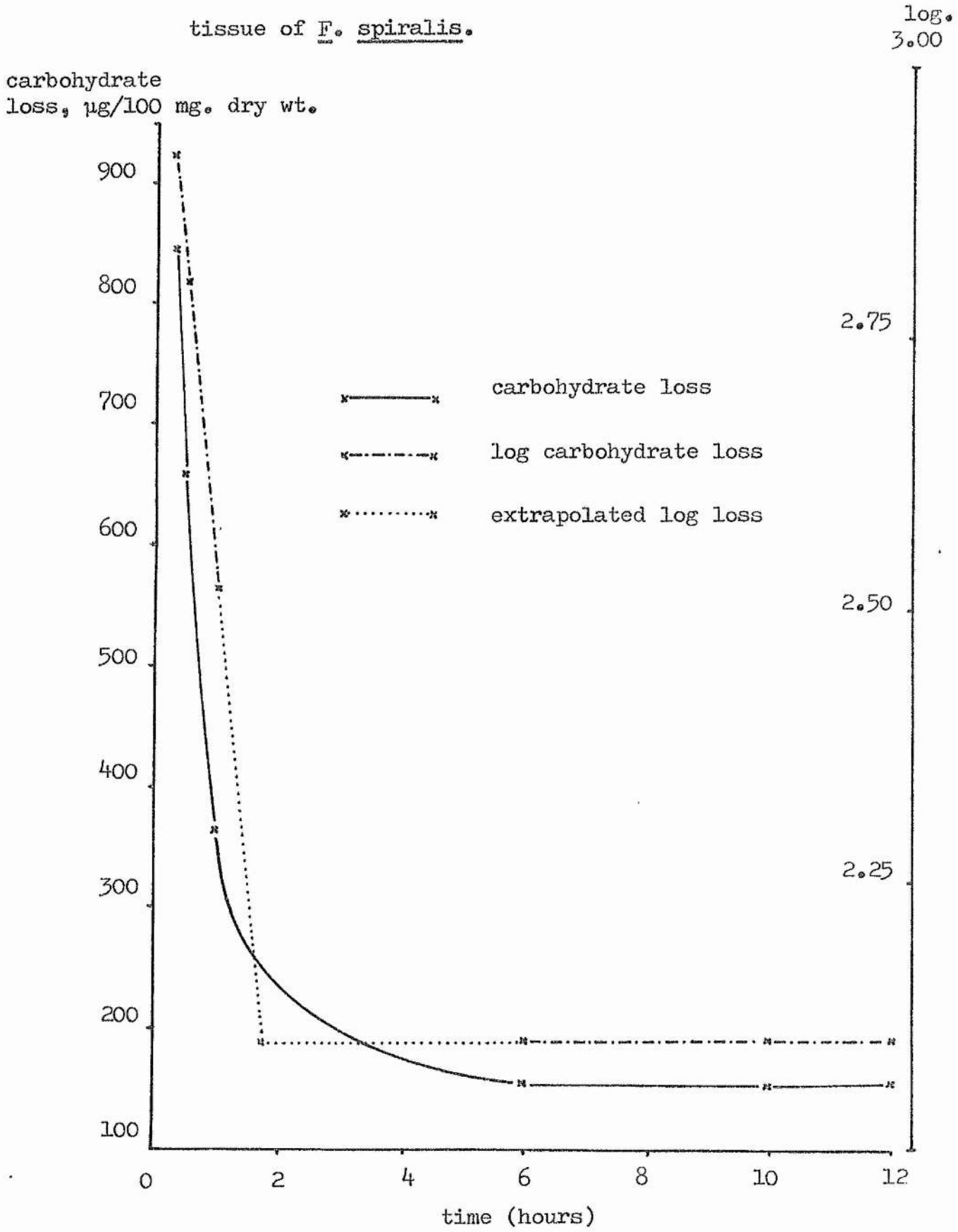
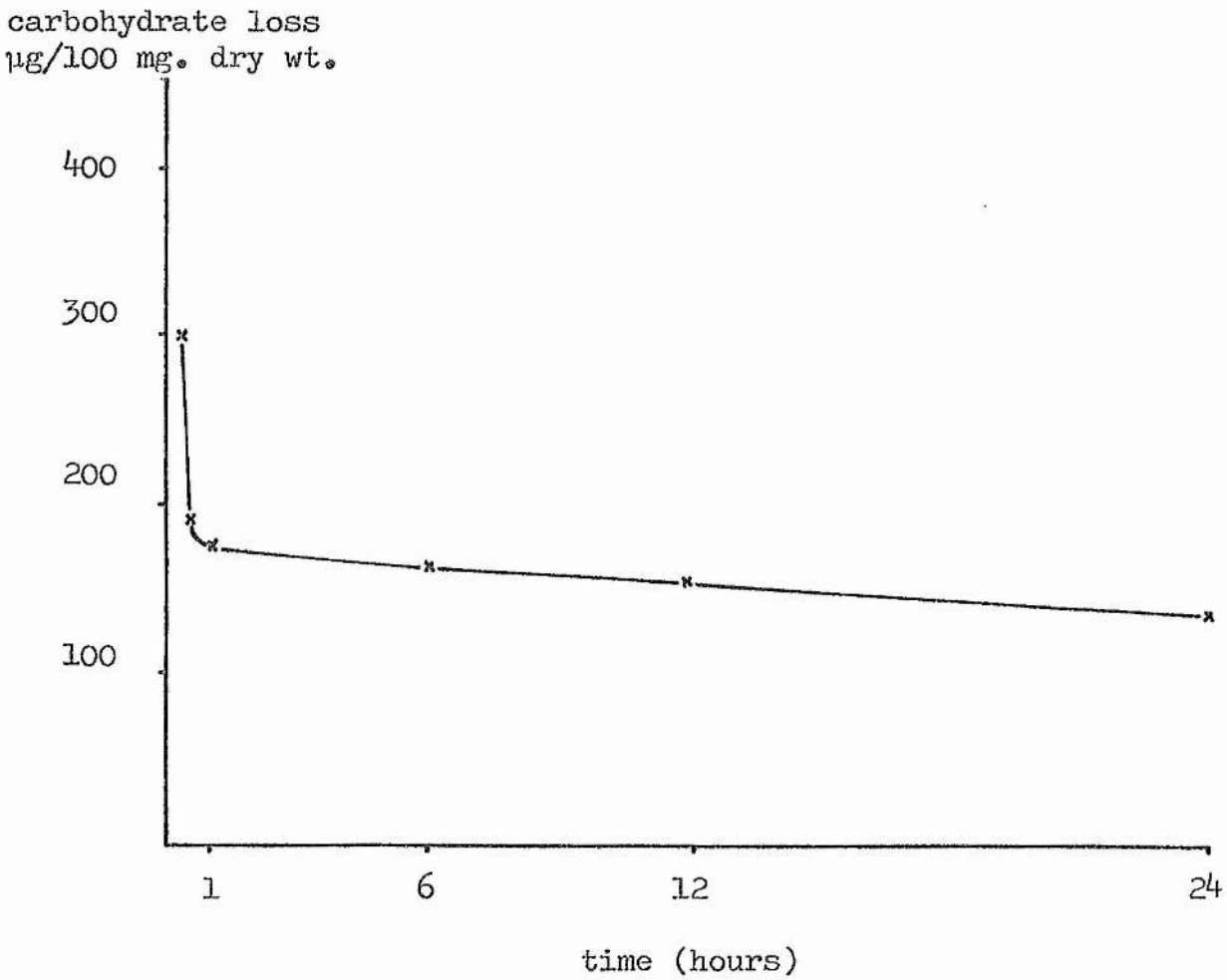


Figure 4:1b. Carbohydrate loss from control incubations of intact thalli of F. spiralis.

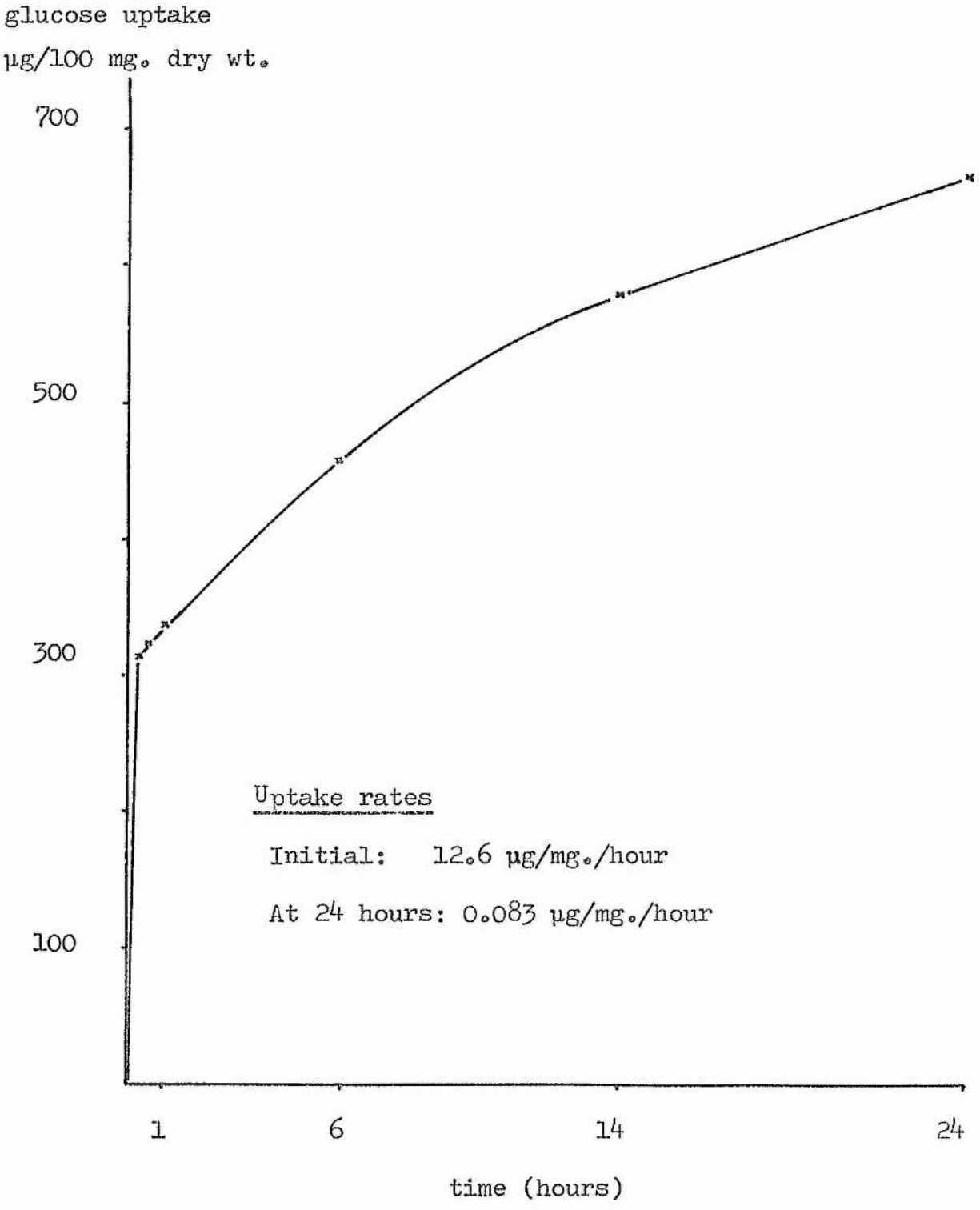


the losses became less appreciable with longer periods of incubation. This presumably indicates that the tissues were able to re-absorb much of the carbohydrate heterotrophically. After 24 hours, when incubations were terminated, entire sporelings and excised apices had both lost approximately 150µg./100mg. dry wt. These results are contrary to those of Sieburth (1969b) who found carbohydrate loss in F. vesiculosus to be photosynthetically linked. Sieburth reports no dark losses by this species. It is important that such losses should be taken into account when considering the efficiency of heterotrophy as a means of carbon fixation.

Allowing for the losses from the controls, figure 4:1b, entire sporelings of F. spiralis showed a gross uptake of glucose from the medium. These results are shown in figure 4:2; the initial rapid phase can probably be attributed to the filling of cell-free spaces, and the subsequent, slower non-linear phase, might represent uptake into the cytoplasm and vacuoles. At 24 hours the rate was 0.083µg. carbohydrate/mg. dry wt./hour and can thus probably be attributed to glucose metabolism, which, as will be seen later, proceeds at a virtually identical rate in this species.

Subsequent washing of these sporelings and analysis of the washing medium indicated that at least 70% of the glucose could be recovered, which is consistent with the results

Figure 4:2. Gross uptake of exogenously supplied glucose by intact thalli of F. spiralis.



of Drew (1969). These results suggest that for the species F. spiralis an overall loss is sustained by thalli in darkness even in the presence of exogenous glucose. Similar experiments in which entire thalli of Pelvetia were incubated in darkness failed to reveal any detectable carbohydrate loss.

2. The effect of glucose concentration on respiratory rate.

Apices of Pelvetia were incubated in media containing 40-4000µg. glucose/ml. filtered sea-water. Respiration was determined manometrically. The results shown in figures 4:3 -4:4a indicate that neither total oxygen consumption or rate of consumption are stimulated by glucose in these concentrations.

The initial oscillation in rate (figure 4:4a), which is expected in any system coming to equilibrium, cannot be attributed to glucose as glucose-free controls exhibited a similar pattern of oxygen consumption. Furthermore, addition of glucose to the control after equilibration similarly had no effect on the respiratory rate (figure 4:4b).

3. Uptake of exogenously supplied glucose-U-^{14}C by thalli of Pelvetia and F. spiralis.

Drew (1969) has reported the varying abilities of eleven species of the Phaeophyceae to take up and metabolize exogenously supplied glucose-U-^{14}C heterotrophically. Only

Figure 4:3. The effect of glucose concentration on oxygen uptake by excised apical tissue of Pelvetia.

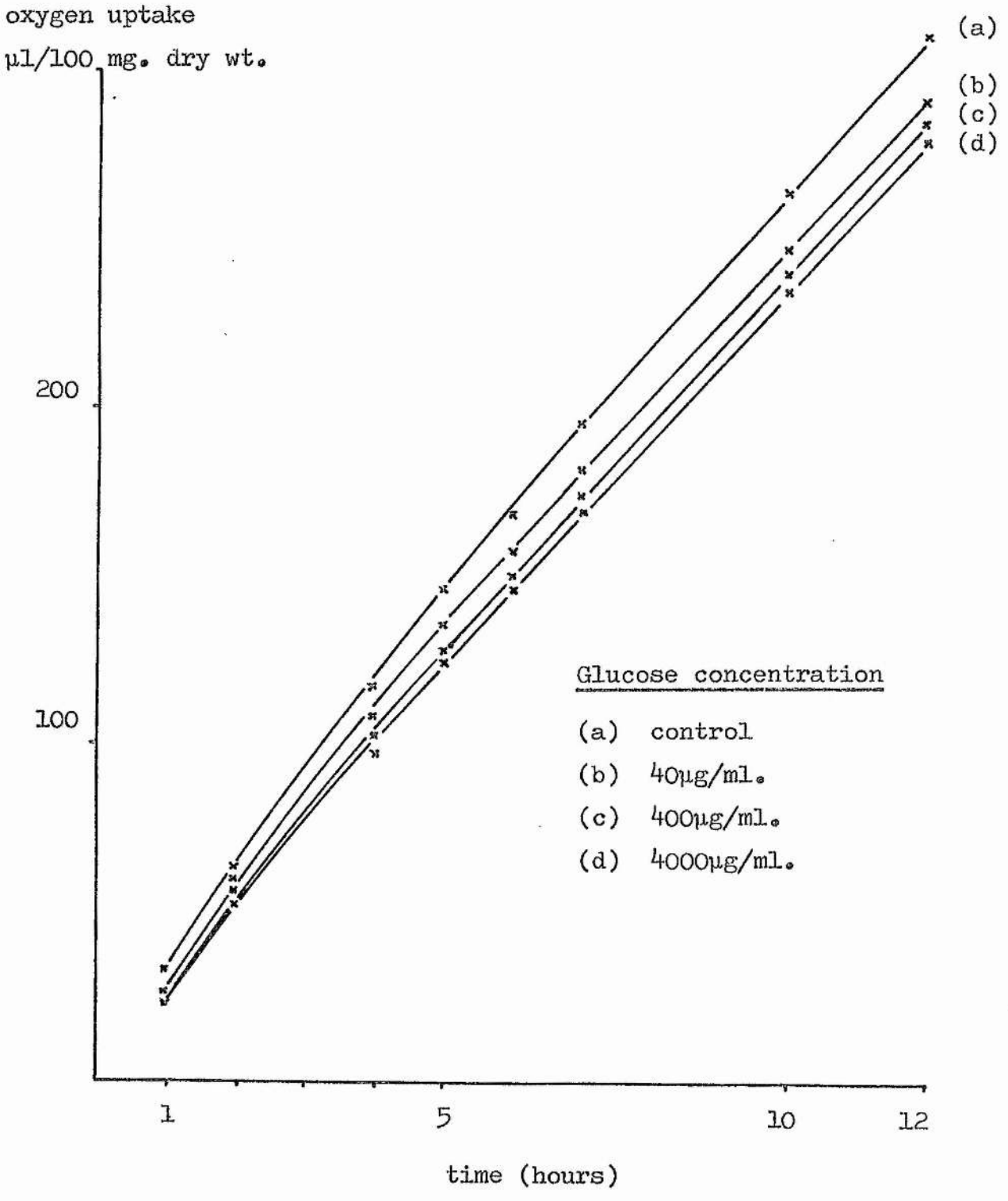


Figure 4:4a. The effect of glucose concentration on the rate of oxygen uptake by excised apical tissue of Pelvetia.

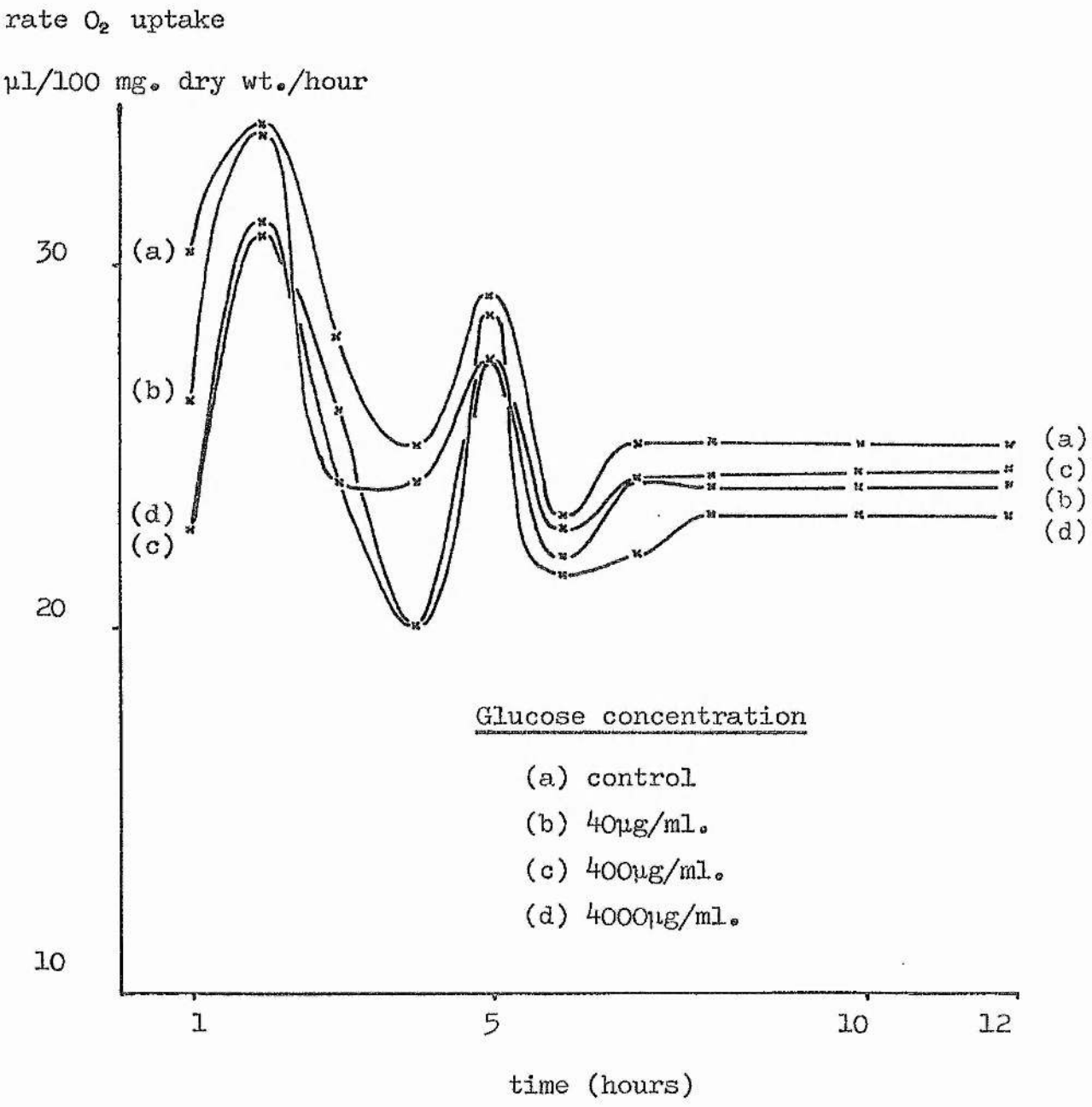
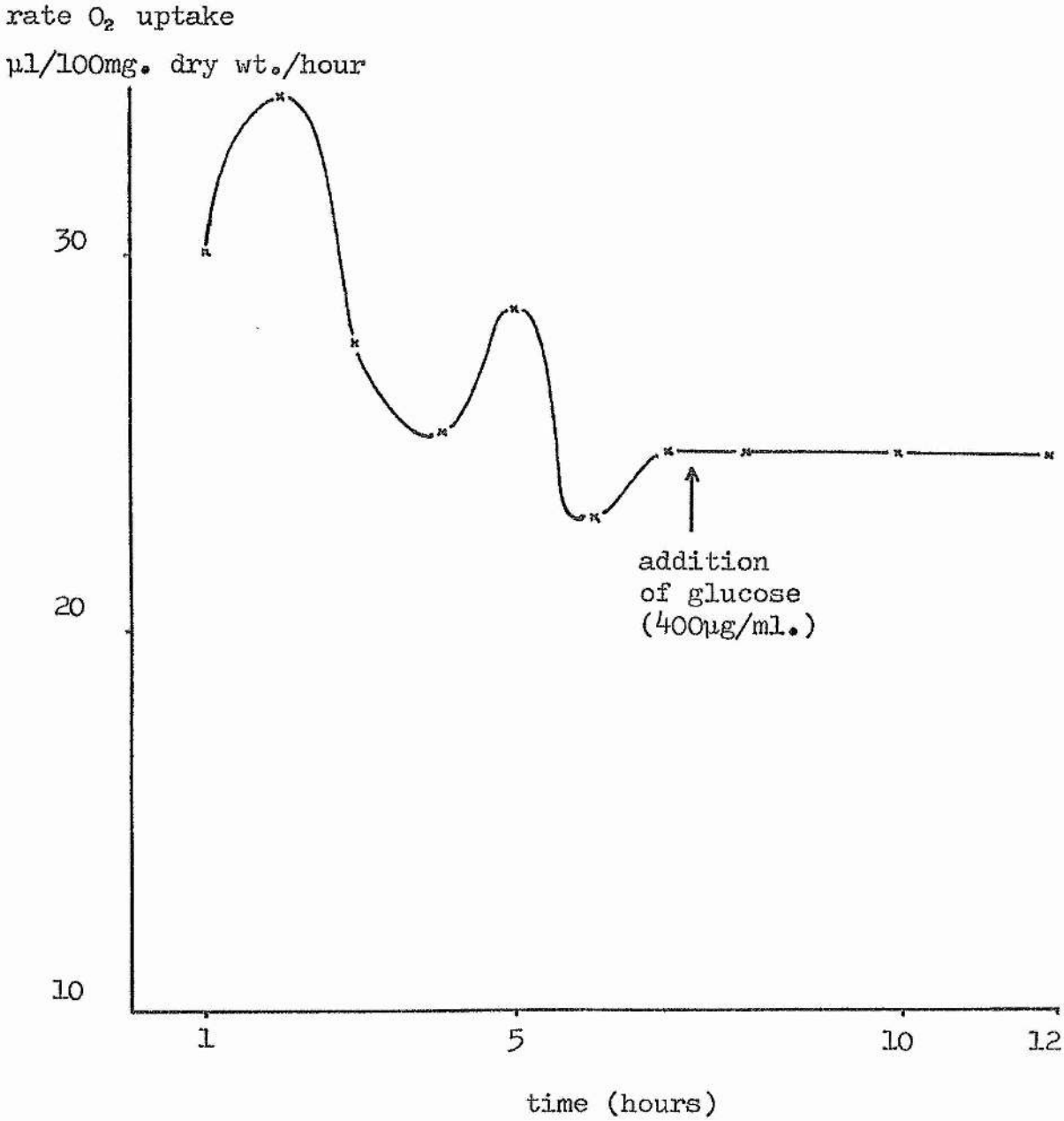


Figure 4:4b. The effect of glucose on control incubations after equilibration.



in Pelvetia and Ascophyllum, two species in which glucose was incorporated into mannitol, was the process of glucose uptake found to be continuous. These results have been verified for apical tissues of these species and for F. spiralis.

Previous work on the heterotrophic metabolism of macrophytic algae has been concentrated on excised apical tissue or on intact thalli. It was thus decided to investigate the possibility of regional variation in the ability to accrete exogenously supplied glucose.

6 cm. long thalli of Pelvetia and F. spiralis were selected and each was cut into 6 x 1 cm. lengths. Equivalent portions were then treated collectively for each species. Incubation media contained 2μCi (1.42×10^6 cpm) of glucose-U-^{14}C in 3 ml. of filtered sea-water. Incubation was carried out at 10°C in darkness for 20 hours. Washing and extraction procedures were as described previously.

Results are shown in table 4:1, and these data are represented graphically in figures 4:5-4:7.

Several interesting features of these results are apparent.

(a) An increase in absorbtion rate towards the base, evident in both species, but in Pelvetia there was a marked sub-apical decline in heterotrophic potential.

(b) This regional variation ability to take up exogenous

Table 4:1. Regional variation in the ability of F. spiralis and Pelvetia to accrete exogenously supplied glucose-U-^{14}C in the dark.

		Washing media	EtOH insoluble	EtOH soluble	EtOH insol as % total	$^{14}CO_2$ evolved	Sp. activity of CO_2
F. spiralis	1.	1365	38	205	16	34	0.08
	2.	1282	39	197	17	22	0.06
	3.	1013	97	210	32	28	0.12
	4.	700	368	195	65	211	0.70
	5.	488	780	487	62	346	1.38
	6.	241	5460	2408	69	1224	4.10
Pelvetia	1.	1076	4340	4627	48	951	5.20
	2.	635	2169	4872	31	779	3.65
	3.	522	2349	6132	28	743	3.58
	4.	441	2269	7059	24	988	4.25
	5.	111	3853	8070	32	912	3.94
	6.	61	8479	8609	50	1536	9.00

Note: (1) The figures 1-6 denote 1 cm. lengths of tissue in basipetal order.

(2) Data for radioactivity are given as cpm/mg. dry extracted wt. of alga.

(3) Specific activities are quoted as cpm/mg. dry wt./µg. C respired.

Key to figures 4:5 and 4:6.

——— Total activity after washing

– – – – Ethanol insoluble fraction

—·—·— Ethanol soluble fraction

····· Washing medium (i.e. elutable fraction)

—··—·· $^{14}CO_2$ released in respiration

N.B. Ordinates are log scale.

Figure 4:5. Regional variation in the ability of thalli of Pelvetia to take up exogenous glucose-U-^{14}C.

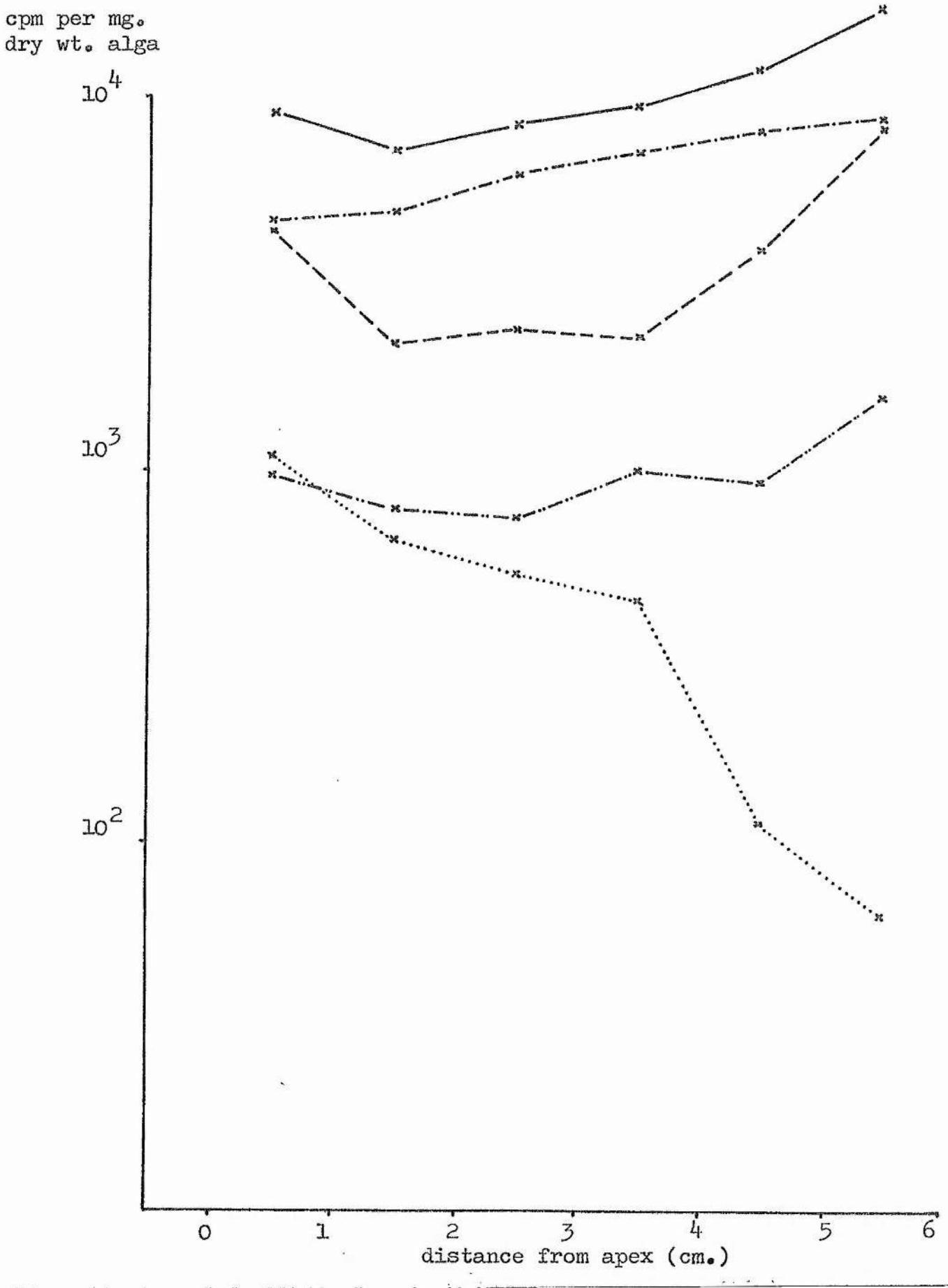


Figure 4:6. Regional variation in the ability of thalli of *F. spiralis* to take up exogenous glucose-U-^{14}C.

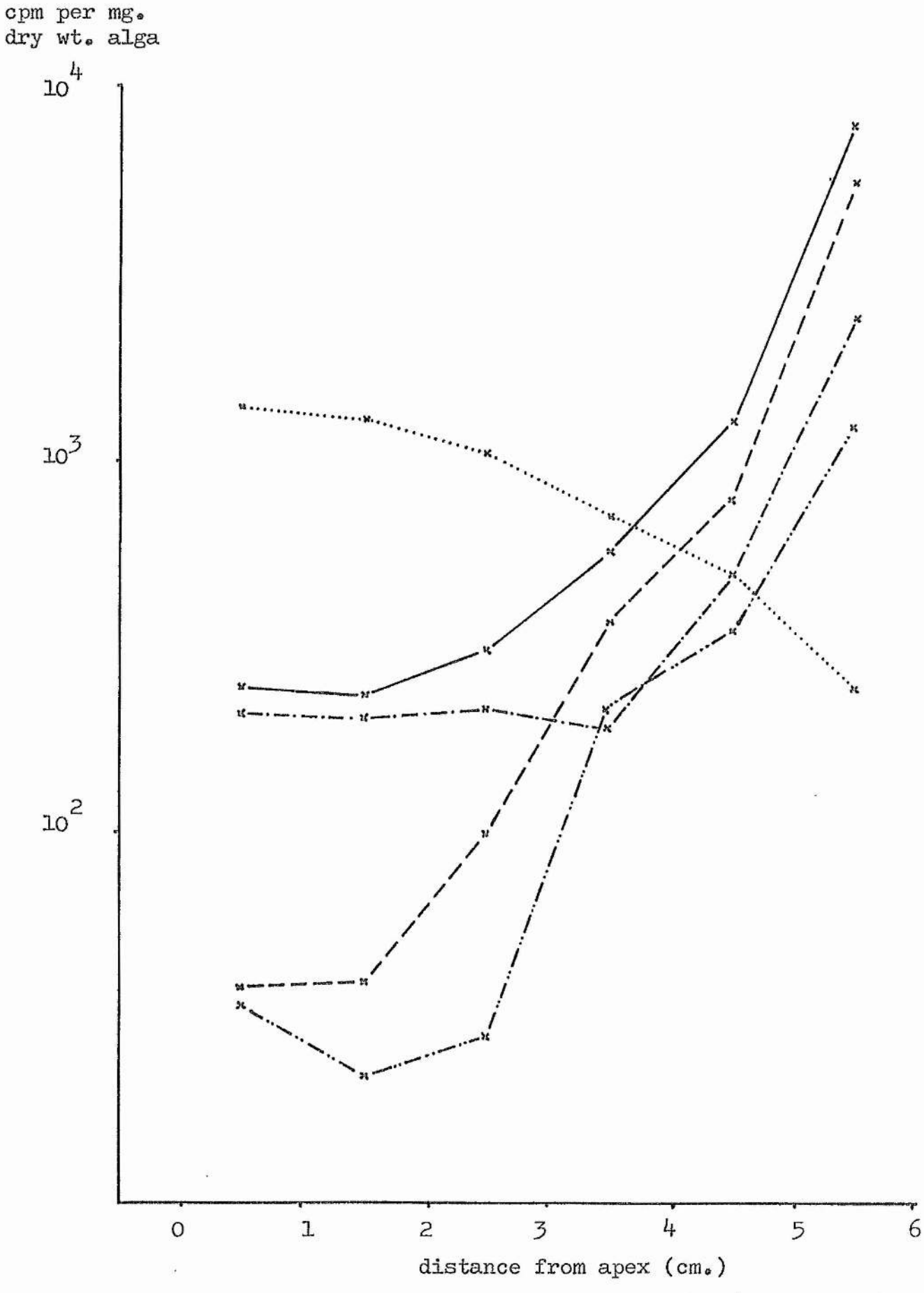
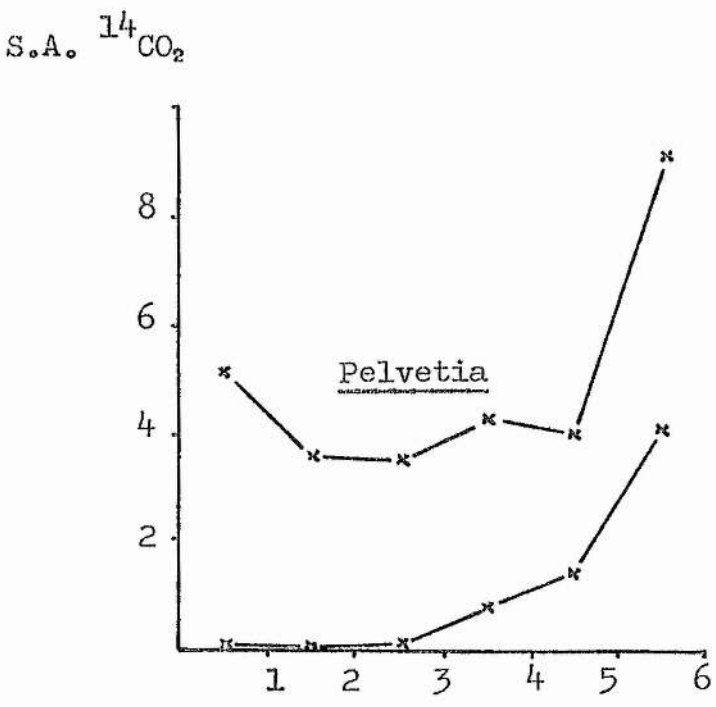
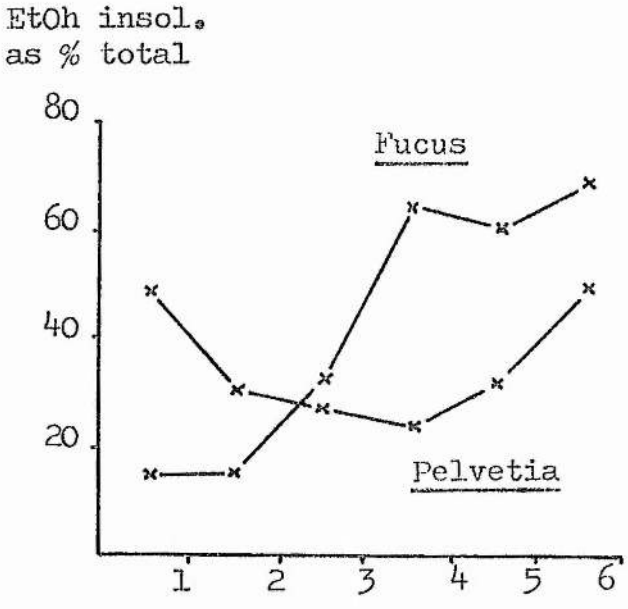


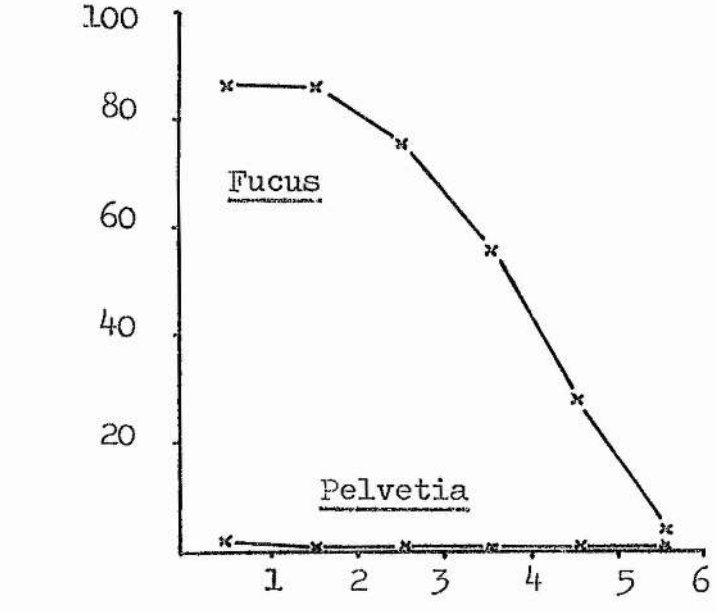
Figure 4:7. Regional variation in the ability of F. spiralis and Pelvetia to accrete exogenous glucose-U-^{14}C.



(a) Specific activity of $^{14}CO_2$ released in respiration.



(b) Ethanol insoluble as % total after washing.



(c) Elutable fraction as % total activity taken up.

Washing media (i.e. elutable fraction) as % total uptake.

	Pelvetia	Fucus
1.	1.07	84.9
2.	0.83	84.5
3.	0.58	76.7
4.	0.45	55.4
5.	0.09	27.8
6.	0.04	3.0

glucose is reflected in the amount of glucose present in the washing media (i.e. elutable fraction), which showed a basipetal decline in both species. Figure 4:7 (c) shows the elutable fraction expressed as % of total activity taken up. Whereas the values for Pelvetia were 1% or less, throughout the plant, those for Fucus show a marked decline towards the base from 85-3%.

(c) Uptake into Fucus apices was very limited and the proportion incorporated into insoluble products was less than in the apical region of Pelvetia. Fucus basal tissue, however, converted 69% of the total radioactivity to insoluble compounds, as compared with 50% in Pelvetia (figure 4:7b).

(d) The specific activity of respired carbon similarly suggests low heterotrophic potential in Fucus apical tissue. It will be seen in Chapter 5, however, that integrity of the thallus is important in this respect, for vastly increased uptakes and specific activities have been recorded using intact thalli.

4. The products of heterotrophic uptake of glucose-U-^{14}C in Pelvetia and F. spiralis.

Bidwell and Ghosh (1963) demonstrated that in (intact?) thalli of F. vesiculosus 80% of the gross uptake of glucose-U-^{14}C remained in the alcohol soluble fraction after 15 hours incubation in darkness, and that 82% of this fraction was present as glucose, which they assumed to be non-metabolised.

Drew (1969) showed that 75% of the gross glucose-U-^{14}C taken up by sub-apical tissues of F. vesiculosus could be eluted, and that this activity was indeed present in non-metabolised glucose. In the previous section of this chapter it was shown that in F. spiralis the proportion of glucose which can be eluted shows a decline towards the base from 85% - 3%. Also, the gross uptake by excised basal tissue was comparable to that by Pelvetia. In Pelvetia and Ascophyllum nodosum low values for radioisotope recovery during washing were associated with rapid glucose metabolism and the synthesis of mannitol (Drew, 1969). It was thus expected that basal tissue of F. spiralis might similarly synthesize mannitol heterotrophically.

Apical and basal tissue of F. spiralis and Pelvetia was incubated with shaking for 24 hours in darkness at 10°C in 3 ml. sea-water media containing 2μCi of glucose-U-^{14}C. After incubation the tissues were washed for 1 hour at 0 - 2°C with shaking. Alcohol extracts were prepared as described previously and were analysed by paper and gas-liquid chromatography. Radioactivity was located on paper chromatograms using a manually operated thin-end window Geiger-Muller tube with 2mm. collimator slit width.

Analysis indicated that in both Fucus and Pelvetia the washing media contained only glucose. This is equivalent

to the non-metabolised glucose suggested by Bidwell and Ghosh (1963) and demonstrated by Drew (1969).

In the alcohol soluble extract of F. spiralis apical tissue no conversion to any other metabolite was evident, C^{14} being located only in glucose.

Contrary to expectations, basal tissue of F. spiralis also showed no labelling of mannitol. However, about 50% of the label was located in a compound having and R_g = 0.25 in ethyl methyl ketone (EMK); glacial acetic acid; saturated boric acid (9:1:1), as seen in figure 4:8. In tertiary butanol; picrate; water solvent this compound ran with glucose. Also, a positive colour reaction was achieved with alkaline $AgNO_3$, suggesting the compound to be a carbohydrate. Evidence of identity was not established beyond this.

Radiochromatograms of the alcohol soluble extracts from Pelvetia showed the major peak of activity to have a mobility 87% of that of an authentic mannitol marker in EMK solvent. Chemical identity was not fully reliable due to the size of the carbohydrate spot and also to the presence of amino acids which corresponded exactly to the peaks of activity. These findings suggested that, contrary to the results of Drew (1969), amino acids and not mannitol might be the products of heterotrophic glucose assimilation in this species. Further investigations proceeded as follows:-

Figures 4:8 - 4:10. The products of heterotrophic uptake of glucose-U-^{14}C in Pelvetia and F. spiralis.

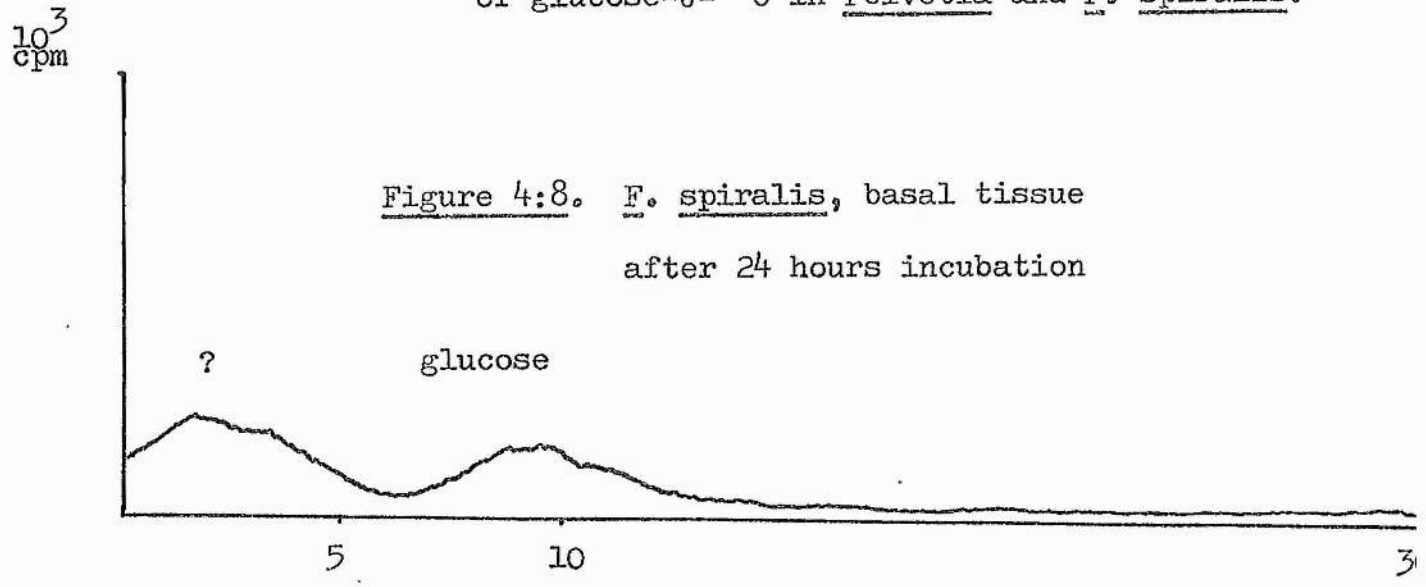


Figure 4:8. F. spiralis, basal tissue after 24 hours incubation

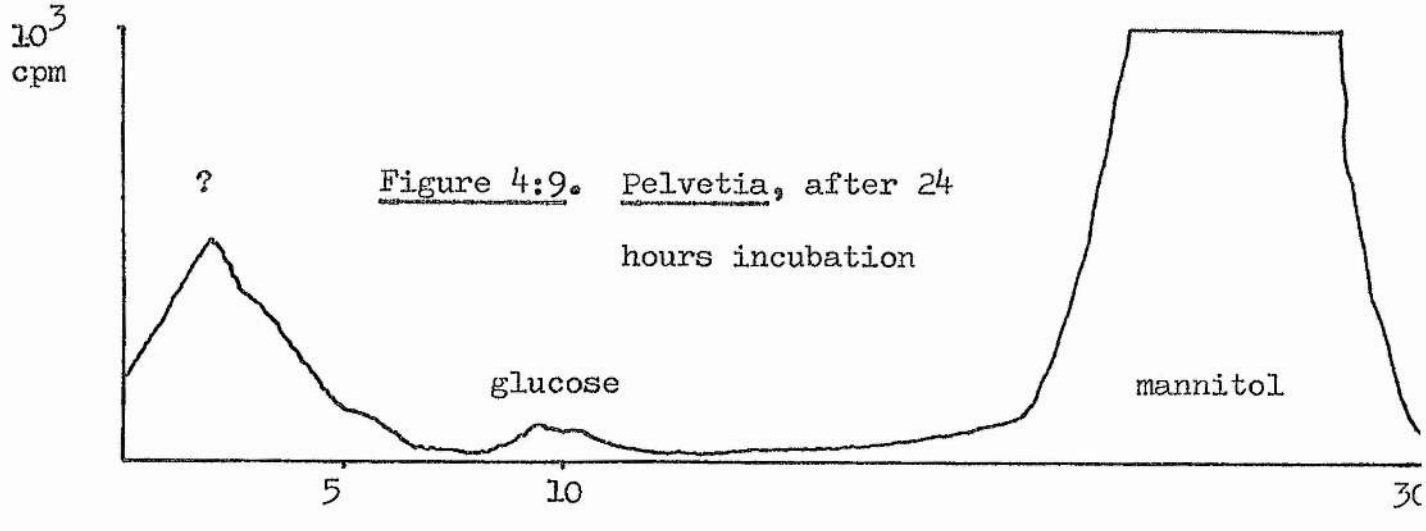


Figure 4:9. Pelvetia, after 24 hours incubation

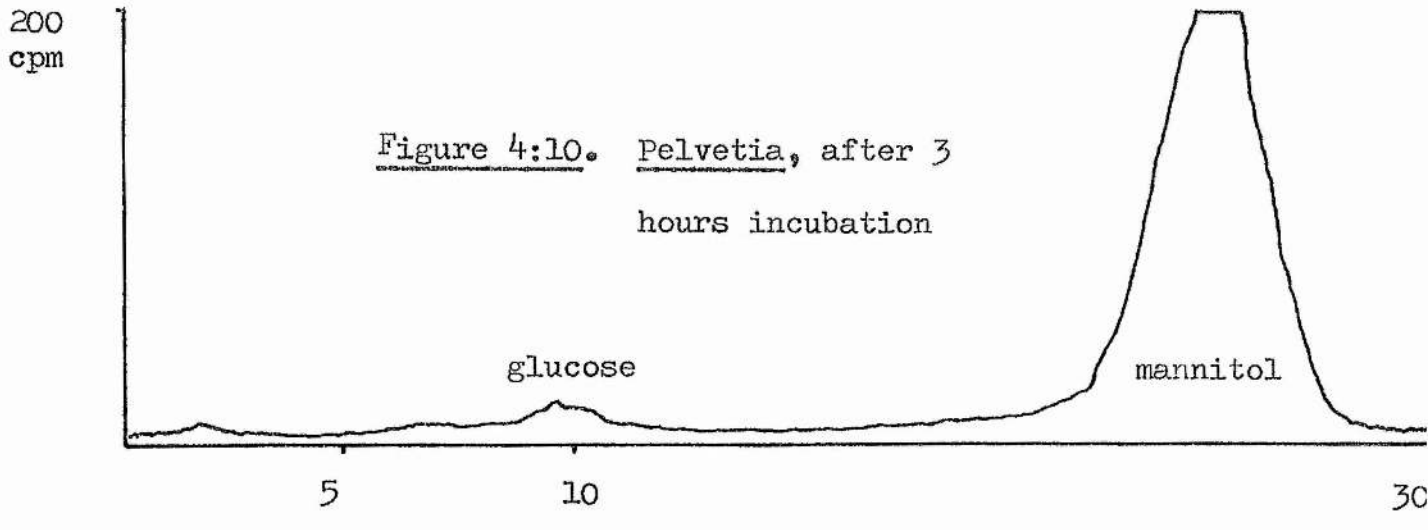


Figure 4:10. Pelvetia, after 3 hours incubation

(i) The alcohol soluble extract was evaporated to dryness and taken up in 1 ml. of 0.1% HCl (pH circa 2) such that amino acids would be dissociated. 100µl was spotted onto Whatman No. 1 chromatography paper, sprayed with ninhydrin and developed at 110°C for 3 minutes. A duplicate spot was assayed for carbohydrate by spraying with alkaline $AgNO_3$. The spots were then assayed for radioactivity.

(ii) 0.5 ml. of this acid solution was introduced into a glass column (15 x 0.5cm. i.d.) containing Amberlite IR-120 (H) cation exchange resin, and flushed with 7.5 ml. of distilled water the effluent should contain undissociated molecules, such as sugars, whilst dissociated amino acids should remain on the resin). The effluent was evaporated to dryness and made up to 0.5 ml. in 0.1% HCl. Radio- and chemical assay was as described previously in (i) above.

(iii) The column was then flushed with 7.5 ml. of 2N-NH_3; of this effluent the first 1.5 ml. was evaporated to dryness and made up to 0.1 ml. in water.

(iv) The subsequent 6.0 ml. was similarly treated, but made up to 0.4 ml. in water. Analysis was as described previously.

The results are shown in table 4:2. These data demonstrate that in sample (i) both carbohydrates and amino acids were present, as expected. In sample (ii) the effluent

Table 4:2. The products of heterotrophic uptake of glucose-U-^{14}C in Pelvetia.

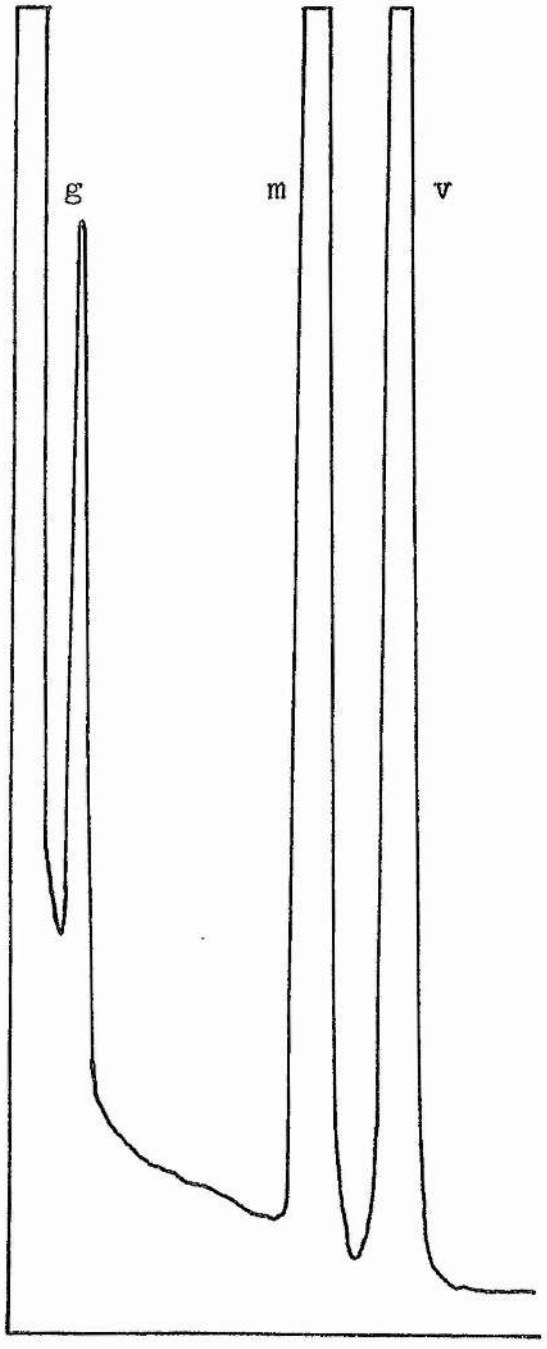
Sample	ninhydrin reaction	cpm above background	% activity
(i) Initial in 0.1% HCl	+ ve	332	100
(ii) IR 120(H) H_2O elution	- ve	284	86
(iii) IR 120(H) 1.5ml. 2N NH_3 elution	$\frac{-}{+}$ ve	2	0.6
(iv) IR 120(H) 6.0ml. 2N NH_3 elution	+ ve	12	3.6
		total recovery	90.2%

contained only carbohydrate and this contained 79% of the total sample (i) activity. Rechromatography (in EMK) and radioassay of this sample indicated the activity to be located in mannitol, the mobility of this peak now corresponding exactly with that of the authentic labelled marker. The results were thus in accord with those of Drew (1969) and indicate mannitol to be the major soluble product of heterotrophic glucose assimilation in Pelvetia. The importance of clearing extracts prior to chromatography in the EMK solvent system must be stressed.

The unidentified compound found in F. spiralis basal tissue was present also in Pelvetia after 24 hours incubation (figure 4:9), but not after 3 hours incubation (figure 4:10). The compound could not, therefore, be a mannitol precursor.

Bidwell and Ghosh (1963) in their studies of the metabolism of glucose-U-^{14}C by F. vesiculosus demonstrated the presence of label in glycerol after 1 hour. However, taking into account the relatively low levels of radioactivity used in the present study, the amount expected would have been below the level of sensitivity of the detection methods used. However, GLC analysis of alcohol soluble extracts did show glycerol to be present as a dark metabolic product in sub-apical and supra-basal tissue of Pelvetia (figure 4:11). Approximately 1.7 mg. glycerol/100 mg. dry extracted weight

Figure 4:11. GLC scan showing the occurrence of glycerol as a dark metabolic product in supra-basal tissue of Pelvetia.



g = glycerol

m = mannitol

v = volemitol

was found in supra-basal tissue. Had this been a product of glucose-U-^{14}C metabolism, carbon-14 would surely have been detected in it. This was not the case, so that glycerol is more likely to be a degradative product of some endogenous metabolite in Pelvetia.

5. Real and apparent heterotrophic uptake.

(a) The effect of chloroform vapour pretreatment on the uptake of exogenous glucose-U-^{14}C by apical and basal tissues of F. spiralis and Pelvetia.

In section 4 of this chapter it was demonstrated that in Pelvetia and F. spiralis the major ^{14}C- labelled components of the alcohol soluble fraction were mannitol and glucose respectively after incubation in glucose-U-^{14}C media in the dark. It has similarly been shown that non-metabolized glucose-U-^{14}C was the major soluble component of F. vesiculosus under the same conditions (Bidwell and Ghosh, 1963; Drew, 1969). Thus the possibility exists that for Fucus heterotrophic uptake may be at least partly 'apparent', probably by binding of the monosaccharide to cell wall materials and/or mucilage, or by the activity of epiphytic bacteria and those existing freely in the sea-water media.

Chloroform vapour pretreatment has been shown to increase membrane permeability in both fungi and algae (Quillet and Legrand, 1952; Drew, 1969). Drew further used the treatment

in an unsuccessful attempt to expose the monosaccharide reducing system of F. vesiculosus under heterotrophic conditions. Mannitol was not produced either by this species or by the control, Ascophyllum nodosum, in which inactivation of the system was evident after 30 minutes pretreatment. In view of this it was thought possible to test the hypothesis of binding, using chloroform vapour pretreatment to create a physical system in which no metabolism could occur.

Entire thalli of Pelvetia and F. spiralis were treated in chloroform vapour for 90 minutes prior to incubation of excised apices and bases for 20 hours at 10°C in darkness in 3 ml. sea-water containing 2μCi glucose-U-^{14}C. Methods of subsequent analysis were as described previously.

The results are shown in table 4:3.

Basal tissue of F.spiralis, apical and basal tissue of Pelvetia; (Data for F. spiralis apices will be considered separately.)

In no instance was gross uptake found to be enhanced by chloroform pretreatment. Radioactivity present in washing media, alcohol soluble and insoluble fractions, and in $^{14}CO_2$ released in respiration was consistently lower than in the corresponding controls. Expressed as % gross uptake, however, radioactivity present in the washing media was increased with respect to the controls. It would appear that glucose metabolism has ceased in these tissues, thus making a greater

Table 4:3. The effect of chloroform vapour pretreatment on the uptake of glucose-U-^{14}C by apical and basal tissues of F. spiralis and Pelvetia.

	Washing media	W.M. as % gross uptake	Net uptake i.e. EtOH insol + sol	Nature of EtOH sol. ^{14}C	EtOH insol as % total	$^{14}CO_2$ evolved	Sp.activity of CO_2
Fucus	211	63	126	Glucose	13	4	0.02
apex	(1365)	(85)	(243)	Glucose	(16)	(34)	(0.08)
Fucus	70	43	94	Glucose	33	19	0.14
base	(241)	(3)	(7866)	Glucose and unidentified	(69)	(1224)	(4.10)
Pelvetia	185	61	118	Glucose	8	4	0.02
apex	(1076)	(12)	(8967)	Mannitol and unidentified	(48)	(951)	(5.20)
Pelvetia	56	62	35	Glucose	40	6	0.05
base	(61)	(0.35)	(17088)	Mannitol and unidentified	(50)	(1536)	(9.00)

Note: (1) Data fn parenthesis denote uptake by the equivalent region of un-pretreated tissue from Table 4:1.

(2) Data for radioactivity are expressed as cpm./mg. dry wt./20 hours.

proportion of the gross uptake freely elutable. Lack of metabolism is further evidenced by negligible decarboxylation of the glucose offered and by reduced values for the specific activity of CO_2. Also, in the alcohol soluble fraction radioactivity was located only in glucose, never in mannitol or disaccharide. Uptake into this fraction was reduced about a hundred fold, and in each case the % conversion to insoluble compounds was significantly lower than in the controls.

Uptake due to extrinsic factors seems to be minimal in these tissues and can probably be discounted as insignificant. The small amount of decarboxylation which does occur can probably most reasonably be accounted for in terms of respiration by bacteria living freely in the sea-water media.

Apical tissue of F. spiralis.

Again gross uptake was reduced after chloroform pretreatment, but net uptake (i.e. after washing) was similar to those described for the tissues above. However, as the heterotrophic potential of Fucus apical tissue is low, even in the control treatment, this uptake due to extrinsic factors is still important, representing 53% of the net uptake by the control.

As membranes of epiphytic bacteria would be destroyed by chloroform vapour pretreatment simultaneously with those of the alga this uptake cannot be attributed to those bacteria. Also, as the free-living bacteria were not analysed along with

the algal tissue they too cannot be responsible for the net uptake (they may, however, account for the low levels of decarboxylation shown in table 4:3). It is suggested that this 53% of the heterotrophic uptake of glucose-U-^{14}C can probably be attributed to glucose binding by apical tissues of F. spiralis. This will subsequently be termed 'apparent heterotrophic uptake'.

5. Real and apparent heterotrophic uptake.

(b) The effect of D-threo-chloramphenicol (CAP) on respiration and heterotrophic uptake of exogenous glucose-U-^{14}C by apical tissue of F. spiralis.

In section 5(a) it was suggested that 53% of the heterotrophic uptake of glucose-U-^{14}C by apical tissues of F. spiralis can probably be attributed to glucose binding. This has been termed apparent heterotrophic uptake. In that estimate no allowance could be made for glucose metabolism by bacteria. It is probably that some of the remaining 47% represents uptake by micro-organisms, but that this contribution is possibly insignificant.

To eliminate their activity the antibiotic D-threo-chloramphenicol (CAP) was used. CAP is supposedly specific for 50-S subunits of bacterial ribosomes, causing faulty translation of mRNA, thus impairing protein synthesis. Although some bacteria are recalcitrant to concentrations exceeding 200µg

CAP/ml., most are susceptible at concentrations of 10 - 20µg/ml. (Mahler and Cordes, 1967).

It appears, however, that at high concentrations CAP also affects protein synthesis in higher organisms (Billet et al., 1965), and impairs certain energy linked functions of mitochondria (Hanson and Hodges, 1963; Stoner et al., 1964). Hanson and Kruegar (1966) demonstrated a halving of the Q_{O_2}(N) in 3-day etiolated corn shoot mitochondria incubated in media containing 1 mg. D-threo-CAP/ml. P/O ratios were similarly diminished, suggesting reduced phosphorylation and ATP availability. Stoner (1965) has shown the damage to be swelling of the mitochondrial membranes.

Three respirometric experiments will now be described in which low CAP concentrations were used in an attempt to eliminate the bacterial contribution to apparent heterotrophic uptake in F. spiralis.

(i) Mixed apical and sub-apical tissue of F. spiralis was incubated in darkness for 24 hours at 10°C in media containing 17µg CAP/ml., and 10µg glucose/ml. Respiration of the experimental treatment was enhanced as compared with the control, as shown in table 4:4.

(ii) At a higher concentration of CAP, 100ug/ml., this stimulation was not evident; indeed, oxygen consumption was suppressed. The results are shown in table 4:5. Control and

Table 4:4. The effect of D-threo-chloramphenicol (CAP) on respiration of F. spiralis.

	Q_{O_2} (μl O_2 uptake/mg/hr.)
Control	0.55
17ug CAP/ml.	0.66

Note: The Q_{O_2} for the control treatment is here lower than in subsequent experiments due to the use here of mixed apical and sub-apical tissues.

Table 4:5. The effect of D-threo-chloramphenicol on respiration and heterotrophic uptake by *F. spiralis*.

	Q_{O_2}	µg C released in resp.	$^{14}CO_2$ evolved (cpm/mg. d. wt.)	Sp. activity of CO_2	EtOH sol. (cpm/mg. d. wt.)	EtOH insol.
Control	0.75	183	17.2	0.09	33.6	82
100µg CAP/ml.	0.50	177	6.2	0.03	13.2	31

Note: (1) Low counting efficiency was here due to the use of a thin end-window Geiger-Muller tube and Ecko scaler (Type N530 F).

(2) The alcohol insoluble fraction expressed as % net uptake is high due to an extended (5 hour) washing period.

experimental incubation media contained 0.2μCi glucose-U-^{14}C/ml. Total CO_2 released in respiration was very similar in the control and experimental treatments, but the specific activity of the carbon was considerably lower in the presence of CAP.

As the algal apices represent a large tissue volume relative to that of any bacteria present, either freely or as epiphytes, it is probable that the majority of O_2 consumed and CO_2 released in respiration was due to algal metabolism. Although the total carbon released in respiration was relatively unaffected by the presence of CAP, the specific activity of this carbon was only one third that in the control. This difference could therefore, be due to the elimination of bacterial respiration. The residual activity might be due to respiration of recalcitrant bacteria, to algal respiration, or to a time lapse for the onset of effectiveness of the drug. But, just as $^{14}CO_2$ released in respiration was suppressed by a factor of 2.8, carbon accretion into the alcohol soluble and insoluble fractions both show suppression by a factor of 2.6. Thus it seems likely that CAP affects heterotrophic uptake by the alga per se, hence the diminished specific activity of CO_2 released in respiration.

(iii) In experiments (i) and (ii) enhancement and suppression of O_2 uptake were demonstrated at CAP concentrations of 17 and

100µg/ml. respectively.

The results in experiment (ii) also suggest an adverse effect on glucose uptake in algal tissues treated with CAP.

A third experiment was conducted in which apical tissues of F. spiralis were pretreated for 23 hours in CAP of varying concentrations prior to dark incubation for 24 hours at 10°C in 3 ml. sea-water media containing both CAP (at the concentration used in pretreatment) and 2µCi glucose-U-^{14}C. The control was 'pre-treated' in sea-water, and 2µCi glucose-U-^{14}C were added simultaneously with that added to the experimental treatments. The results are shown in table 4:6.

As in experiment (ii) of this series the results indicate reduced glucose-U-^{14}C uptake into the algal tissues. This suppression increased with CAP concentration. This is consistent with a hypothetical decrease of ATP availability, ATP being required for active glucose transport. The results of Ellis (1964) and Hanson and Kruegar (1966), which demonstrated uncoupling of oxidative phosphorylation and decreased P/O ratios in corn shoot mitochondria in the presence of CAP, strongly support this hypothesis.

Diminished specific activity of the CO_2 released in respiration is consistent with suppression of glucose-U-^{14}C uptake. However, gross CO_2 release was enhanced with increase

Table 4:6. The effect of D-threo-chloramphenicol pretreatment on respiration and heterotrophic uptake of exogenously supplied glucose-U-^{14}C in F. spiralis.

	Q_{O_2} (i) (µl/mg.d.wt./hr)	Q_{O_2} (f)	total O_2 uptake, µl	CO_2 rel. in resp, µl.	R.Q. CO_2/O_2	$^{14}CO_2$ evolved	Sp. activity of CO_2	washing media	EtOH sol. + insol.
Control	0.7	0.7	10.66	10.0	0.94	34	6.35	175	38
10µg	0.9	1.2	14.19	65.1	4.65	38	1.08	116	36
20µg	0.9	1.0	15.02	100	6.65	15	0.28	94	29
50µg	0.6	0.6	11.21	155	10.34	14	0.17	74	22
100µg	0.6	0.4	10.42	250	24.00	14	0.10	88	22

Note: (1) A thin end window G.M. tube and Ecko scaler were again used.

(2) Q_{O_2} (i) and Q_{O_2} (f) are initial and final quotients respectively.

(3) Specific activities are high because in this experiment non-respiratory CO_2 was collected in a 10% KOH blank and subtracted from gross CO_2 collected.

in CAP administered, which strongly suggests a shift from the exogenous to an endogenous respiratory substrate.

It is possible to account for enhanced R.Q. in two ways:

(1) The increased $^{12}CO_2$ could derive from the decarboxylation of a large pool of substrate, such as organic acid, which is more highly oxidised than carbohydrate.

(2) Enhanced R.Q. could also be due to aerobic fermentation, which is known to be caused by uncouplers.

In aerobic fermentation, oxidation of the substrate is incomplete so that less CO_2 is produced per substrate molecule (Gibbs, 1962), but this, in terms of 'R.Q.', is only partly compensated by diminished oxygen uptake at high CAP levels.

In order to account for enhanced gross CO_2 output in terms of aerobic fermentation it is necessary to hypothesize increased turnover of substrate molecules. As mentioned previously, uncoupling action and decreased P/O ratios are consistent with lack of ATP, and hence greater availability of ADP and P_i, thus releasing the normal rate limitation on glycolysis. This effect, as seen here in apical tissue of F. spiralis, is similar to the Pasteur effect in that carbohydrate is not being 'spared'. The system requires that respiration is rate controlled at the initial stages where carbohydrate enters the glycolytic sequence.

Confirmation of these hypotheses would require analysis for organic acids and for the products of fermentation.

The pattern of oxygen uptake is exactly analagous to the known effects of 2:4 dinitrophenol and other uncoupling agents, which remove rate limitations on oxidation.

A secondary effect which could explain the decrease in oxidative metabolism would be the decreasing functional efficiency of mitochondria with rising CAP levels, for example through decreased ATP availability to the mitochondria. This explanation is consistent with Stoner's (1965) observations of mitochondrial swelling induced by CAP. Also, the explanation reasonably explains why this system can run down whilst glycolysis, being extramitochondrial, can be enhanced.

The supply of glycolytic intermediates (phosphorylated sugars) for residual oxidative phosphorylation and for the postulated fermentation could still be maintained by 'substrate phosphorylation', ATP being formed from the stages

diphosphoglyceraldehyde ⟶ 3 phosphoglycerate

phosphoenolpyruvate ⟶ pyruvate

α-ketoglutarate ⟶ succinate

It is interesting in the context of the above hypothesis that the first and second stages are extramitochondrial.

In conclusion, all the evidence is consistent with CAP acting primarily as an uncoupler of oxidative phosphorylation.

This could be critically tested by direct assay for ATP.

Because of these effects due to CAP it was not possible to determine the bacterial contribution to glucose utilization. It is probable, however, that using algae washed in freshly filtered sea-water the contribution due to epiphytes can be dismissed as negligible. Similarly, the use of freshly filtered sea-water media should reduce the number of free-living bacteria to a negligible level.

CHAPTER 5

The Translocation of Metabolites in Pelvetia and F. spiralis.

Contents

Introduction

It was established by various workers during the 19th. century (Will, 1884; Oliver, 1887; Rosenthal, 1890) that some members of the Laminariales (Lessoniaceae), possess cells in their stipes resembling the sieve elements of vascular plants. Parker (1963, 1965b) detected the movement of C^{14} labelled photosynthate along the sieve elements of Macrocystis at rates up to 78cm/hour. No conduction was demonstrated, however, in the medullary trumpet hyphae. In contrast with most vascular land plants, which conduct largely sucrose, Parker (1966) established that mannitol was the major labelled substance translocated along the sieve elements of Macrocystis.

In response to earlier work by higher plant physiologists, Parker (1956) studied the translocation of P^{32} and fluorescein in Laminaria agardhii and Fucus vesiculosus.

He found no evidence for $P^{32}O_4^{=}$ translocation and was the first to doubt the validity of Fritch's (1945) assumption that trumpet hyphae function in conduction in these algae. Anatomical studies by Parker (1956) did not suggest any resemblance between trumpet hyphae and the sieve tubes of higher plants.

Despite these facts it has not been satisfactorily established that Fucus and Laminaria do not conduct carbohydrate along their trumpet hyphae.

In chapters 3 and 4 the following facts were demonstrated:

(a) The apices and bases of Pelvetia and F. spiralis contain different endogenous concentrations of mannitol, and

(b) These tissues exhibit different abilities to take up exogenous glucose heterotrophically.

In view of these results it was proposed to test the possibility of longitudinal conduction in these species.

Experimental

Acropetal and basipetal translocation in Pelvetia and Fucus spiralis.

Translocation experiments were carried out in hermetically sealed 50ml. conical flasks each containing 2μCi (1.42×10^6cpm.) of glucose-U-^{14}C in 3ml. of sea-water. Each flask contained a single entire thallus 6cm. in length and suspended by a cotton thread such that only the apex or base of the thallus was immersed as required. $^{14}CO_2$ released by respiration was collected in 1ml. of 10% KOH in small phials. Flasks were wrapped in foil and the tissue incubated at 10°C for 24 hours in a metabolic shaker such that less than 1cm. of each thallus was wetted by the incubation medium. At the end of this time the thalli were cut into 6 x 1 cm. lengths, and each portion was individually washed in darkness in 3ml. of sea-water at 1-2°C for 1 hour. Washed tissues were extracted with 80% ethanol, and extracts and residues were analysed for radioactivity.

Three such experiments were conducted as follows:

(a) Pelvetia base immersed.

(b) F. spiralis apex immersed.

(c) F. spiralis base immersed.

The results are shown in table 5:1 and in figures 5:1-5:3.

Although uptake into intact tissues is greatly enhanced compared with the uptake into the corresponding excised tissue (table 4:1), it appears that this carbohydrate is not translocated in significant amounts. These results are discussed more fully in Chapter 9.

Table 5:1. Acropetal and basipetal translocation; the distribution of ^{14}C after the selective incubation of attached apices or bases.

		Washing media	EtOH insoluble	EtOH soluble	EtOH insol as % total	$^{14}CO_2$ evolved	Sp. activity of CO_2
Pelvetia	1.	14	40	133	23	-	-
	2.	8	33	135	20	-	-
	3.	0	36	115	24	-	-
	4.	10	29	103	22	-	-
	5.	34	105	132	44	-	-
	R 6.	830	18600	10367	57	26000	91
F.spiralis	R 1.	628	2560	451	84	3800	6.8
	2.	251	531	114	82	-	-
	3.	15	9	12	43	-	-
	4.	6	5	23	17	-	-
	5.	0	3	20	13	-	-
	6.	10	6	4	57	-	-
F.spiralis	1.	6	21	46	31	-	-
	2.	0	14	33	30	-	-
	3.	0	13	24	35	-	-
	4.	0	23	27	46	-	-
	5.	0	7550	3289	66	-	-
	R 6.	69	27650	9947	67	24500	35

Note: (1) For each treatment 1-6 denote 1cm. lengths of tissue from apex to base.
(2) R indicates that portion of tissue immersed in isotope.
(3) Data for radioactivity are given as cpm/mg. dry extracted weight of alga.
(4) Specific activities are quoted as cpm/mg. dry extracted wt./µgC respired.

Key to figures 5:1 - 5:3.

_____ Total activity after washing

------- Ethanol insoluble fraction

- - - - Ethanol soluble fraction

••••••• Washing medium (i.e. elutable fraction)

N.B. Ordinates are log scale.

Figure 5:1. Acropetal translocation in Pelvetia.

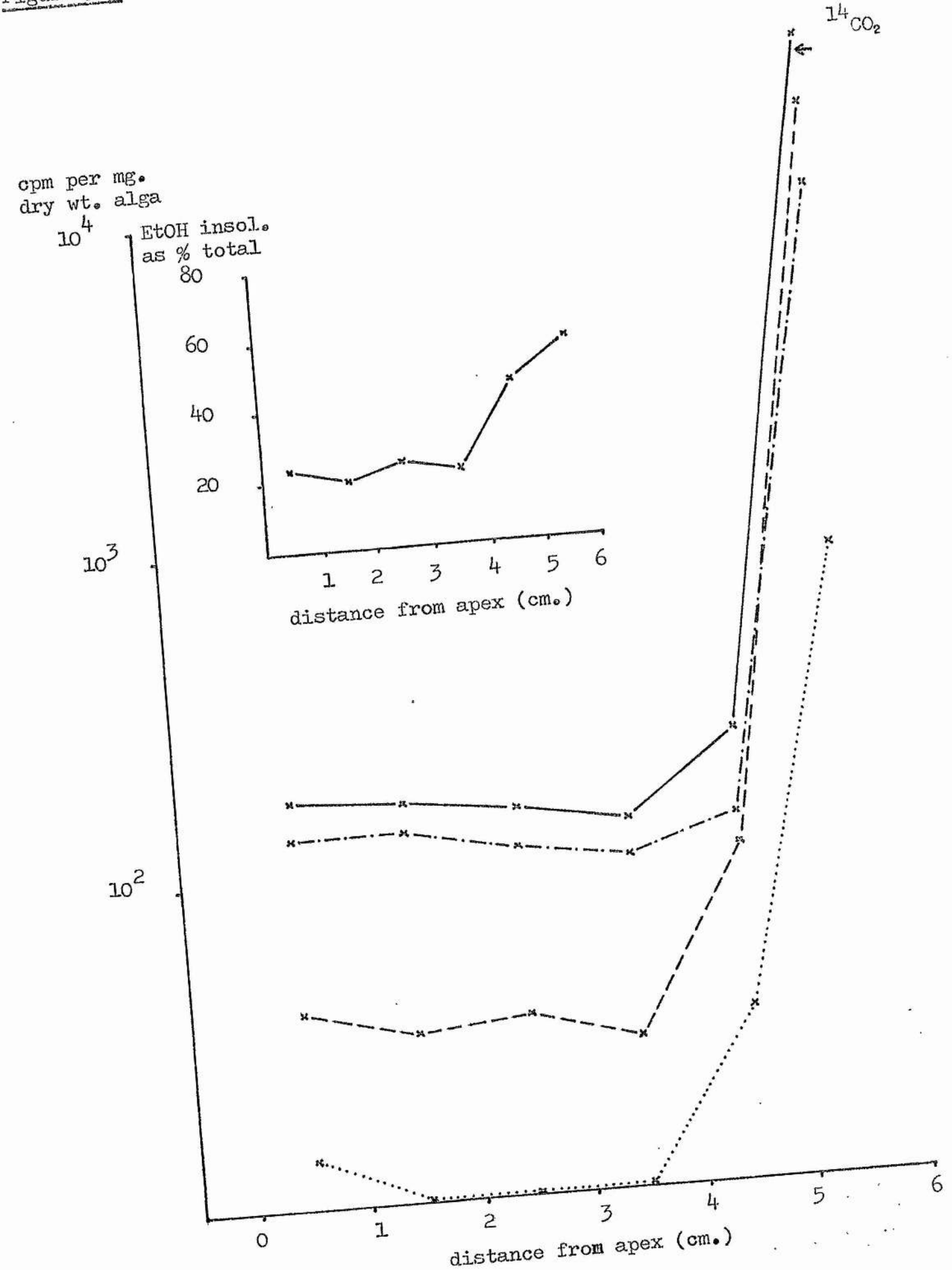


Figure 5:2. Basipetal translocation in F. spiralis.

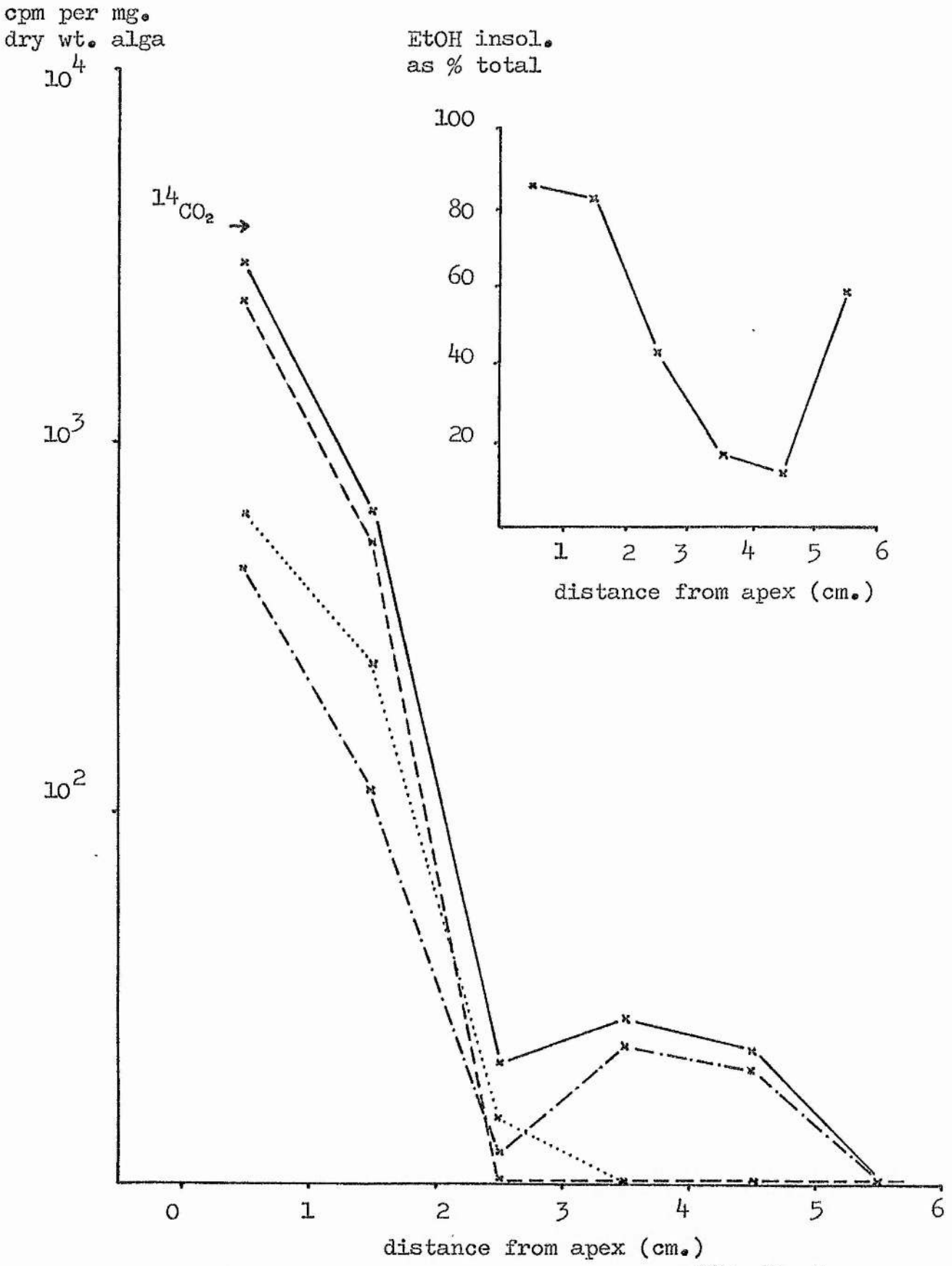
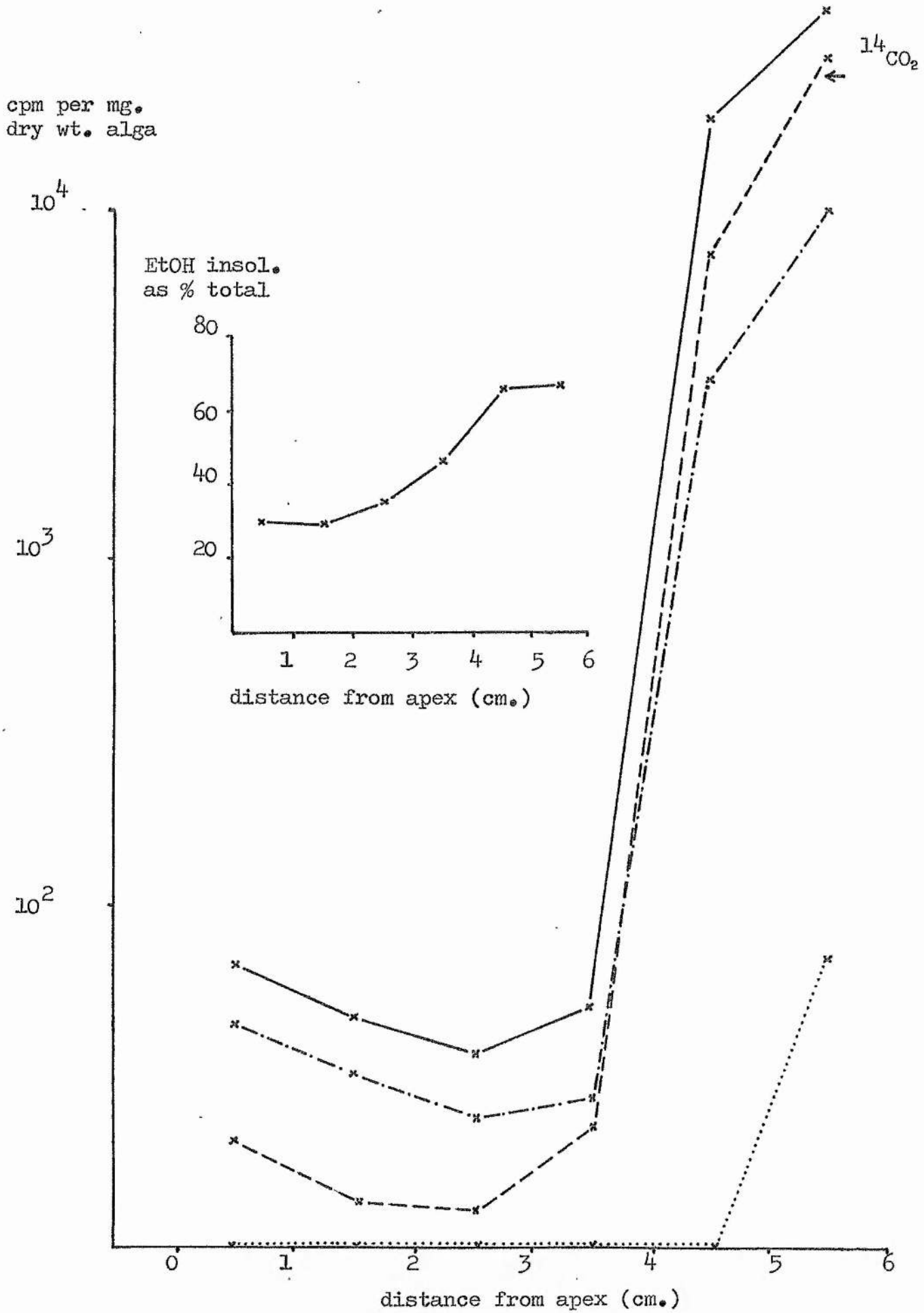


Figure 5:3. Acropetal translocation in F. spiralis.



CHAPTER 6

Respiratory pathways in F. spiralis and P. canaliculata, and the heterotrophic synthesis of mannitol.

Contents page

Introduction

1. Algal respiratory pathways

Studies concerning algal respiratory pathways have largely been confined to unicellular organisms. The latter are thus the main subject matter of this review, but where possible reference is made to the macrophytic algae.

The Embden-Meyerhof-Parnas (EMP) pathway.

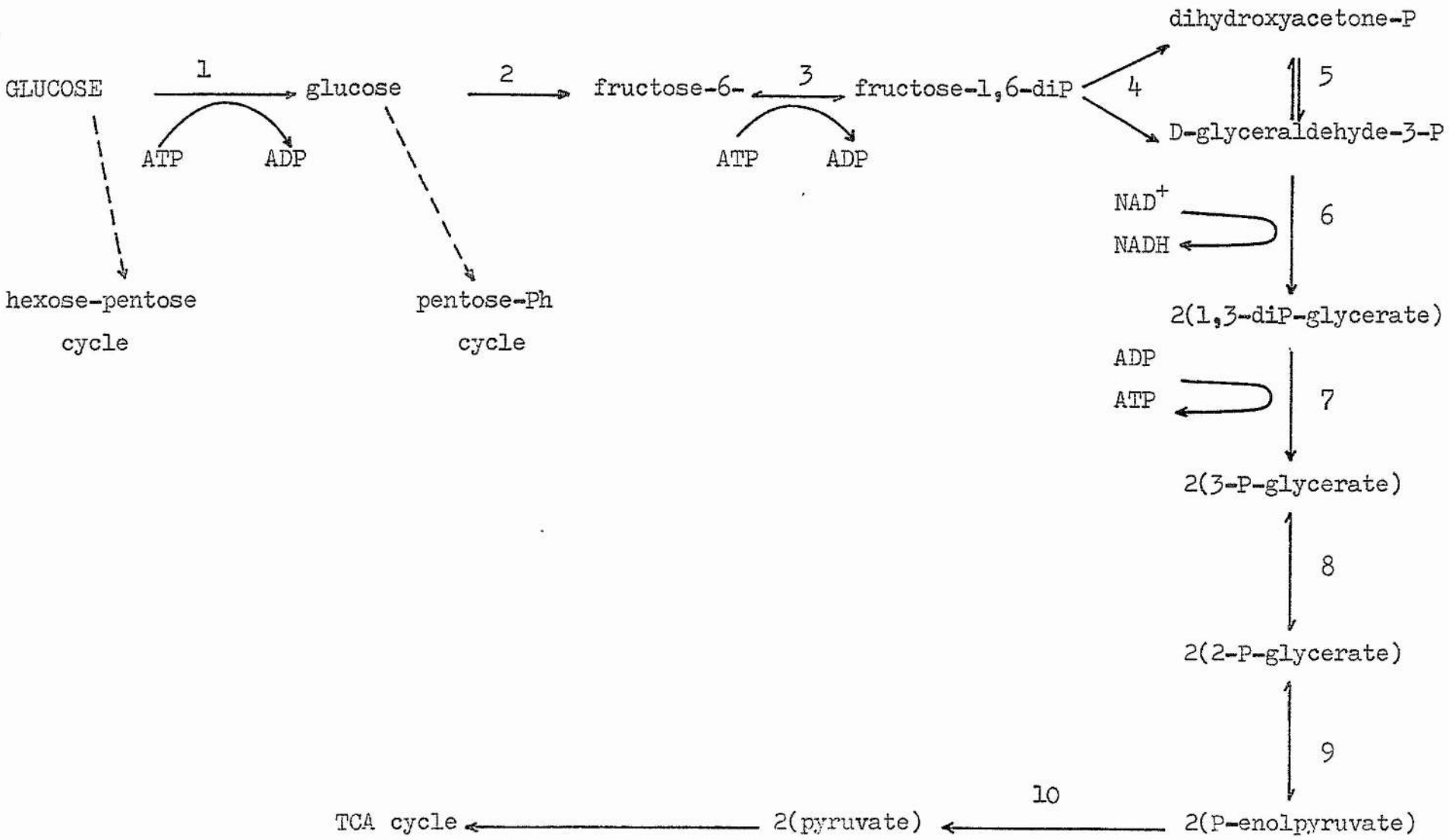
Evidence for the operation of this pathway, which is considered as the metabolic sequence from glucose to pyruvate (figure 6:1) ,has been derived by several means; the detection and utilization of postulated intermediates; the presence of enzymes catalyzing postulated reactions; the sensitivity of some of these enzymes to specific inhibitors; and the degradation of specifically labelled glucose to products labelled as predicted.

The postulated intermediates of the EMP pathways were identified in Chlorella pyrenoidosa by Moses et al. (1965), and accumulation of pyruvate was observed in the same species after arsenite treatment by Kandler (1955). Similar results were obtained in Ochromonas malhamensis by Reazin (1956), who further offered glucose-1- or -2-^{14}C to this species in the presence of arsenite and found the specific activity of pyruvate formed to be half that of the initial glucose. Also, glucose-1-^{14}C gave methyl labelled pyruvate, whereas

Enzymes of the EMP pathway.

1. hexokinase
2. phosphoglucose isomerase
3. phosphofructokinase
4. (fructose-P) aldolase
5. triose phosphate isomerase
6. triose phosphate dehydrogenase
7. phosphoglycerate kinase
8. phosphoglyceromutase
9. enolase
10. pyruvate kinase

Figure 6:1. The Embden-Meyerhof-Parnas scheme of glycolysis.



glucose-2-^{14}C produced α-labelled pyruvate. These facts are all in accord with the operation of the EMP pathway. $^{14}CO_2$ recovery from equal quantities of respired glucose-1- and -6-^{14}C showed the ratio of label from C-1 to that from C-6 in Ochromonas (Reazin, 1956) and Chlorella (Kandler and Gibbs, 1959) to be unity, suggesting that the EMP pathway might possibly be the only pathway of glucose respiration in these algae.

Enzyme preparations also lend support to the operation of the EMP pathway, at least in vitro, but this does not necessarily indicate in vivo activity.

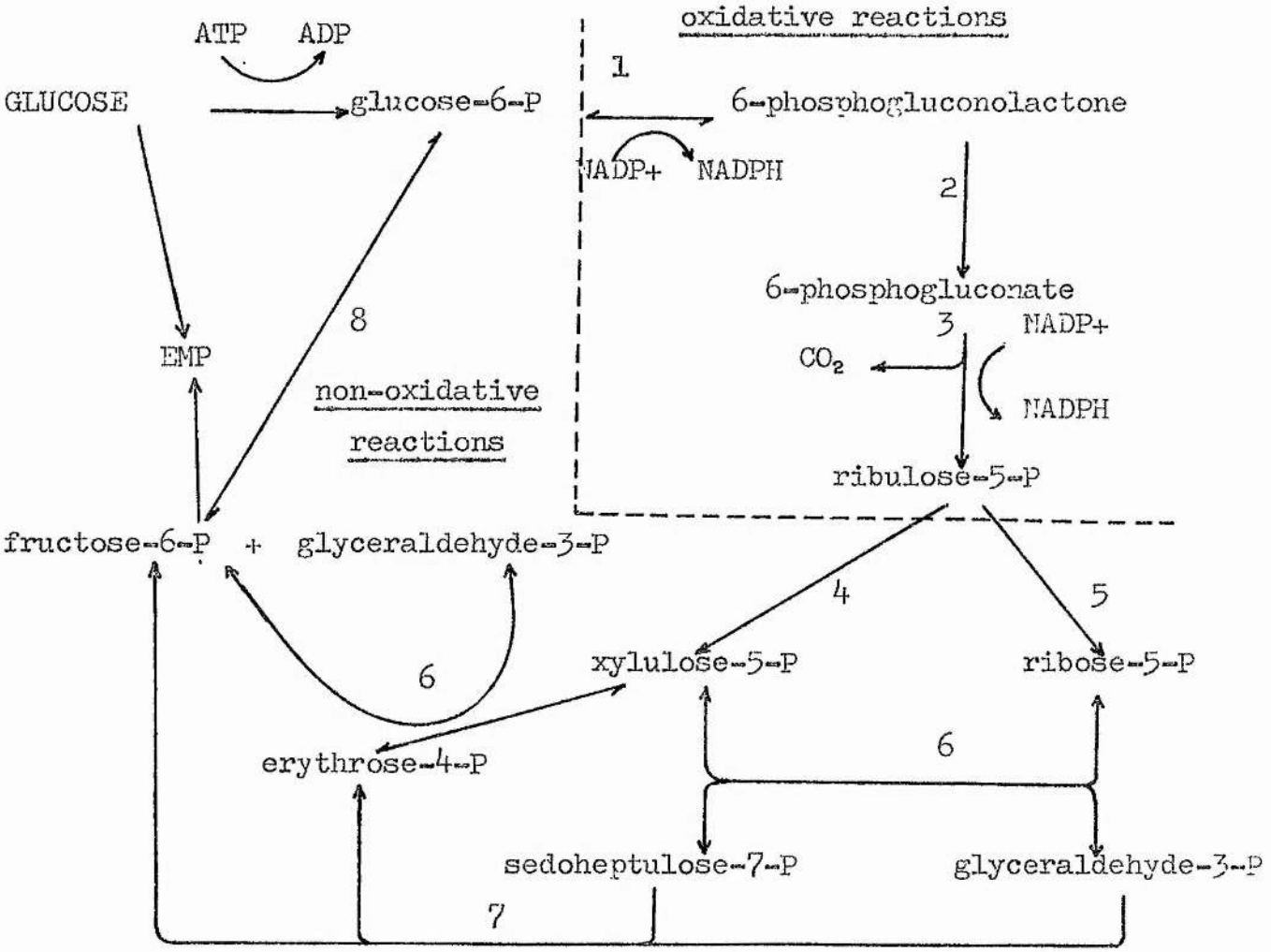
It appears that the glycolytic sequences in marine green algae are similar to those in higher plants. Several of the enzymes have been identified in both the Chlorophyceae and the Rhodophyceae (for a review, see Jacobi, 1962), but in the Phaeophyceae the extraction of functional enzymes is complicated by the presence of polyphenolic compounds which inhibit enzyme activity (see Chapter 7).

The Pentose-Phosphate (P.Ph.) pathway.

The complete reaction sequences of the P.Ph. pathways are shown in figure 6:2.

Although the enzymes of this pathway have been detected there is no evidence for the in vivo operation of the complete pathway (Gibbs, 1962). Indirect evidence has been obtained by

Figure 6:2. The Pentose-Phosphate pathway, and its relationship with the EMP pathway.



Enzymes of the pentose phosphate pathway

1. glucose-6-P dehydrogenase
2. lactonase
3. 6-phosphogluconate dehydrogenase
4. ribulose-5-P epimerase
5. phosphoriboisomerase
6. transaldolase
7. transketolase
8. phosphohexoisomerase

Richter (1959) who detected glucose-6-P dehydrogenase in extracts of Anacystis nidulans. Fructose diphosphate aldolase was not detected, which further lends support to carbohydrate breakdown via the P.Ph. pathway in this species.

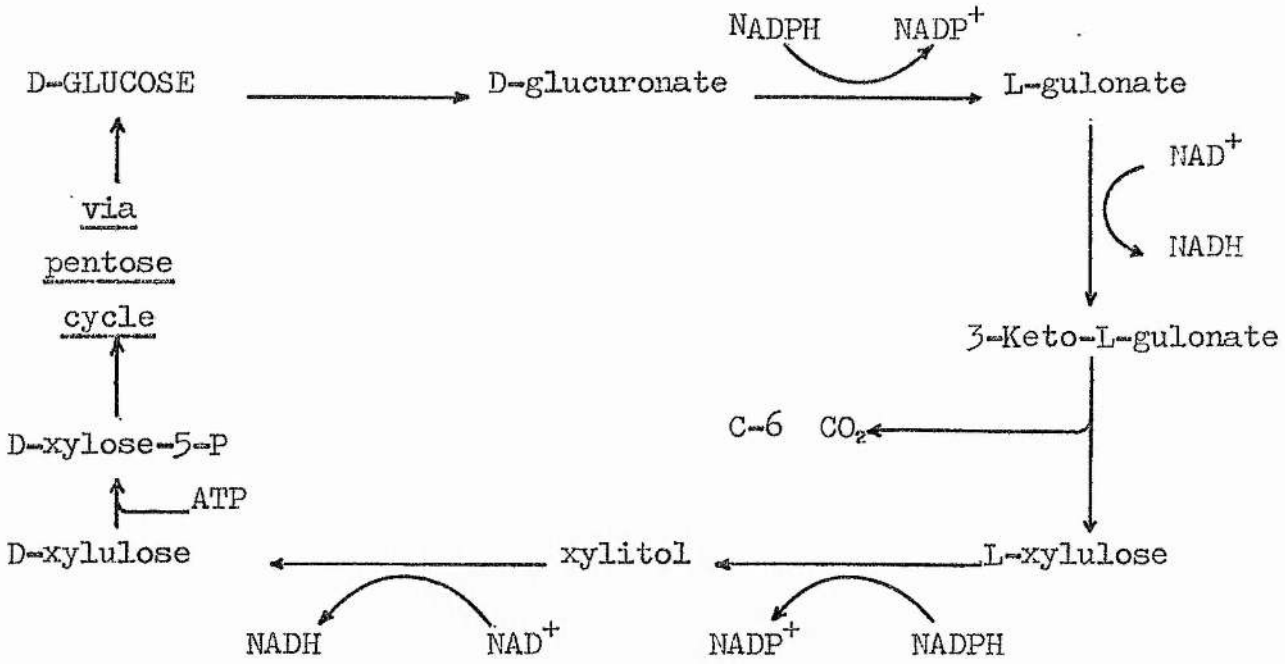
The Hexose-Pentose cycle.

This cycle, which is characterised by the participation of non-phosphorylated intermediates and the release of C-6 of the hexose chain as CO_2, was recognised by Gibbs (1959) in certain animal and plant systems. C-1 of L-gulonate is lost as CO_2, but since in the formation of gulonate from glucuronate there is total inversion of the hexose carbon skeleton, this is equivalent to C-6 of the originally supplied hexose. As shown in figure 6:3 the cycle continues via the non-oxidative sequences of the P.Ph. pathway, and is differentiated from the oxidative reactions of the latter by the loss of C-6 instead of C-1 as CO_2.

The lack of glucose-6-P dehydrogenase and enzymes of the EMP pathway in certain Rhodophyceae (Jacobi, 1958) is the only indication of the possibility of this cycle in algae. This possibility could be critically tested by using glucose-1- and -6-^{14}C substrates, in which case the ratio of C-6/C-1 $^{14}CO_2$ would be expected to exceed unity.

In the red alga Iridophycus flaccidum, Bean and Hassid (1956) have reported the direct oxidation of non-phosphorylated

Figure 6:3. The hexose-pentose (glucuronate-xylulose) cycle.



Note: (a) The reaction sequence between D-glucose and D-glucuronate is actually more complex, involving uridine phosphates.

(b) In the two coupled oxidoreductive reactions the reductive steps involve NADP and the oxidative steps involve NAD.

(c) In the conversion of D-glucuronate to L-gulonate there is total inversion of the carbon skeleton such that C-1 of glucuronate becomes C-6 of gulonate.

hexoses, yielding the corresponding acids. The fate of the acids was not determined by them, but Lin and Hassid (1969) demonstrated the fate of D-mannuronic and D-guluronic acids in alginic acid synthesis im Fucus gardneri.

The Tricarboxylic-Acid (TCA) or Kreb's cycle.

This cycle, which is considered as the oxidation of pyruvate to CO_2 and water (figure 6:4), is evidenced in growth studies (see Chapter 1), manometric and tracer experiments.

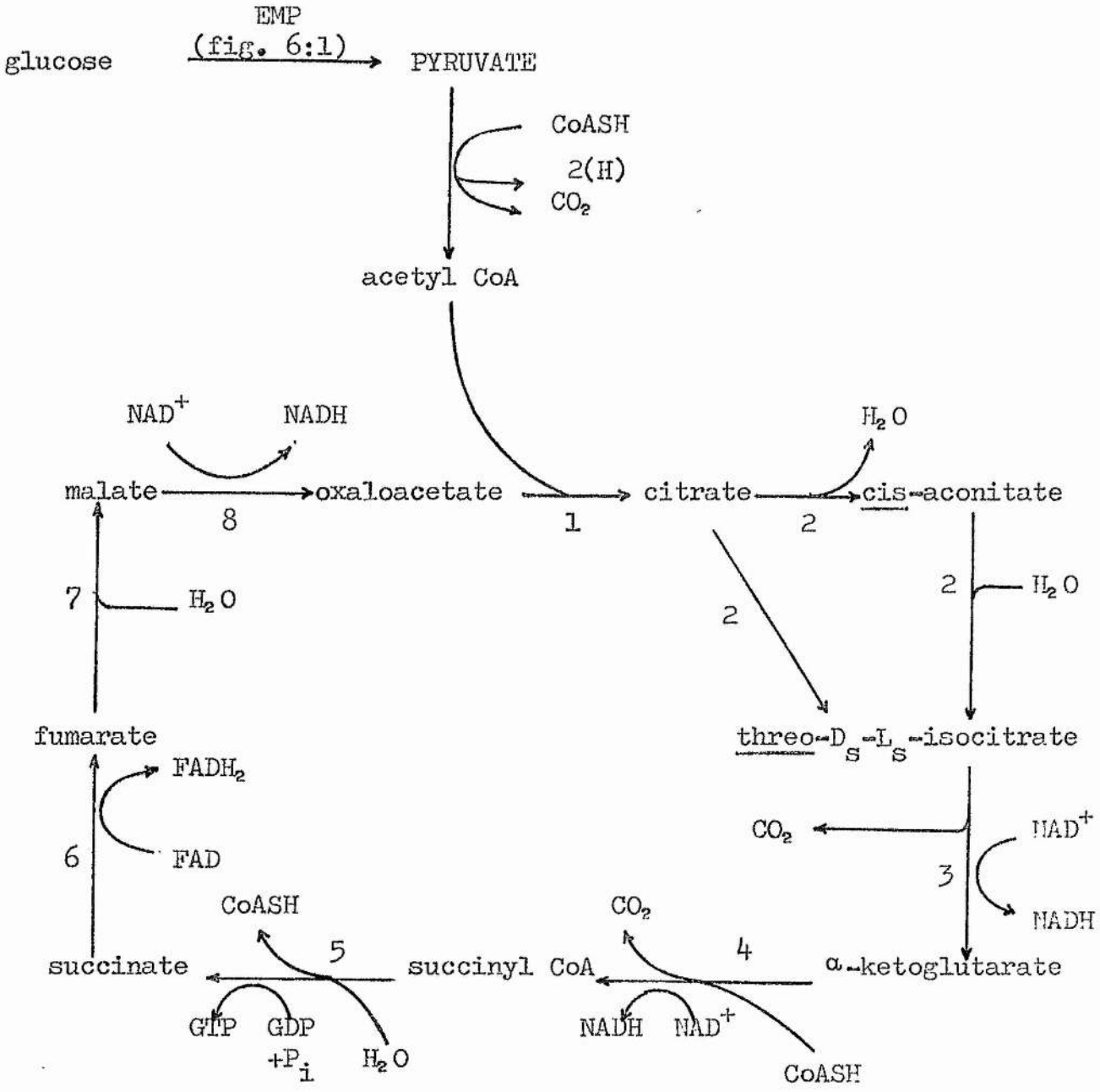
The metabolism of acetate -1- and -2-^{14}C in Chlorella ellipsoida revealed that the carboxyl carbon predominates over the methyl carbon in respiratory CO_2, which is in accordance with the operation of the TCA cycle in this species (Fujita, 1959).

Jacobi (1958) demonstrated the presence of the TCA cycle intermediates pyruvate and α-ketoglutarate in Laminaria saccharina and L. digitata. It is interesting in this context that Bidwell and Ghosh (1963), who have presented evidence for the operation of this cycle in F. vesiculosus by the isolation of labelled intermediates, found acetate-1- and -2-^{14}C to yield $^{14}CO_2$ in very similar quantities. This finding is only in agreement with the functioning of the TCA cycle if total depletion of the initial medium and considerable recycling are postulated. Unfortunately, from the data

Enzymes of the tricarboxylic acid cycle.

1. citrate condensing enzyme
2. aconitase
3. isicitrate dehydrogenase
4. α-ketoglutarate dehydrogenase
5. succinic thiokinase
6. succinate dehydrogenase
7. fumarase
8. malate dehydrogenase

Figure 6:4. The tricarboxylic acid cycle.



presented it is not possible to make assessment of these points.

There is no direct evidence that in algae the TCA cycle is linked to the EMP pathway as it is in higher plants, or indeed, that the enzymes mediating the reactions are mitochondrial.

Quantitative estimation of the pentose-phosphate pathway.

The successful demonstration of enzymes of this pathway has led to considerable speculation concerning its quantitation *in vivo*. There is, however, no direct proof of the operation of this pathway in living algal cells.

Wood *et al.* (1963) have reviewed the methods commonly employed in the estimation of respiratory pathways; these are

(a) using the yield of ^{14}C in triose-P derivatives from glucose-1- and -6-^{14}C.

(b) using $^{14}CO_2$ yields from glucose-1- and -6-^{14}C.

(c) calculations based on ^{14}C randomization in derivatives of glucose-2-^{14}C.

None of these methods has been employed in the estimation of pathways in the Phaeophyceae. Because of the vagaries of intermediary metabolism in algae, attention here is confined to the second method, that of estimation based on $^{14}CO_2$ yields from glucose-1- and -6-^{14}C. The common premise

for measurements of the P.Ph. pathway by this method has been that in the EMP pathway C-1 and C-6 are incorporated equally into triose-P derivatives (including $^{14}CO_2$ released in respiration), whereas in the P.Ph. pathway only C-6 is incorporated into triose-P, since C-1 is converted solely to CO_2. The important assumption in making this premise has been that the ^{14}C of dihydroxyacetone-P and glyceraldehyde -P is equilibrated by the triose-P isomerase reaction. Although triose-P isomerase is known to be an active enzyme (Wood et al., 1963), there is some evidence that equilibration of triose-phosphates may be incomplete (Cahill et al., 1959; Lynn et al., 1960; Rose et al., 1962). However, Katz et al. (1966) have shown that despite incomplete equilibration of ^{14}C in the triose-phosphates, valid results can still be attained for the evaluation of the P.Ph. pathway.

The P.Ph. pathway has been variously defined, often as a model for the calculation of its role in metabolism. The different interpretations of the pathway can be formulated as follows:

1. Pathway *acyclic, pentose-P synthesised by oxidative and non-oxidative sequences.

   glucose-6-P + 2NADP$^+$ ⟶ CO_2 + pentose-P + 2NADPH

   5 Fructose-6-P ⟶ 6 pentose-P

2. Pathway cyclic, fructose-6-P not **recycled to glucose-6-P.

   3 glucose-6-P + 6NADP$^+$ ⟶ 3CO_2 + 5triose-3-P + 6NADPH

---

* Cycling: the resynthesis of hexose-P from pentose-P.

** Recycling: the conversion of fructose-6-P to glucose-6-P which is further metabolised by the EMP or P.Ph. pathway in the same proportion as the original glucose.

or:

$$3\text{glucose-6-P} + 6\text{NADP}^+ \longrightarrow 3CO_2 + 2\text{fructose-6-P} + \text{triose-3-P} + 6\text{NADPH}$$

3. Pathway cyclic, fructose-6-P recycled to glucose-6-P.

$$\text{glucose-6-P} + 6\text{NADP}^+ \longrightarrow 3CO_2 + \text{triose-3-P} + 6\text{NADPH}$$

Early estimations of the P.Ph. pathway did not consider the aspect of recycling of hexose-phosphate and were thus in accord with the second interpretation shown above (Katz et al., 1955; Wang and Krackov, 1962; the latter authors did, however, make provision in their calculations for the drainage of cycle intermediates into anabolic routes). If no recycling of fructose-6-P occurred then the ^{14}C from administered fructose-1- and -6-^{14}C would have no access to the P.Ph. pathway and would be metabolized by the EMP pathway, whence the Bloom and Stetten ratio, C-1 $^{14}CO_2$/C-6 $^{14}CO_2$, would be unity. If, on the other hand, glucose-6-P and fructose-6-P were in complete isotopic equilibrium, ^{14}C of the hexose-6-phosphates would be treated identically such that the ratio of $^{14}CO_2$ yields from glucose-1- and -6-^{14}C should be equal to those from fructose-1- and -6-^{14}C and both should be greater than 1. The results of Merlevede et al. (1963) were consistent with extensive recycling in certain mammalian tissues. A similar approach was employed by Wang et al. (1962), who showed a close agreement between observed and calculated $^{14}CO_2$ recoveries from specifically labelled gluconate substrates.

Little recycling was evidenced by them in tissues of the tomato.

The effect of omitting to consider recycling is to produce conservative results due to the dilution of label in the C-1 position of hexose-P entering the cycle. This is because in the P.Ph. pathway CO_2 will be derived from C-1 of glucose exclusively if (and only if) the hexose-P regenerated by the cycle is constrained from oxidation in favour of the hexose-P added originally, an unreasonable restriction.

Katz and Wood (1960, 1963) have calculated the dilution factor from the ratio of specific activities of the hexose-P pool when glucose-1- and -6-^{14}C substrates are respired. The factor is, however, limited in use to the calculation of pathways using triose-P derivatives from glucose-1- and -6-^{14}C. When using relative CO_2 yields from these isotopes a second unknown is introduced due to the fact that $^{14}CO_2$ derives from both the EMP and P.Ph. pathways whereas, by contrast, triose-P derivatives are assumed to derive from a common pool. This second unknown, which is equivalent to the extent of oxidation of C-3 of glyceraldehyde-3-P to CO_2 has been avoided by Katz and Wood (1963) by the use in their calculations of <u>specific yields</u> of $^{14}CO_2$, defined by Wang and Krackov (1962) as the yield of $^{14}CO_2$ in cpm divided by the activity of utilized glucose expressed as cpm/μ

mole. The equation used by Katz and Wood is:

$$\frac{Gl\ CO_2 - G6\ CO_2}{1 - G6\ CO_2} = \frac{3PC}{1 + 2PC}$$

(for mathematical derivation see Katz and Wood, 1963)

Gl CO_2 and G6 CO_2 are the specific yields of $^{14}CO_2$ from glucose-1- and -6-^{14}C respectively.

PC is the proportion of respiration proceeding via the P.Ph. pathway.

This equation allows calculation of the P.Ph. pathway, but does not differentiate between the EMP and non-triose phosphate pathways. If one assumes the EMP and P.Ph. pathways to operate exclusively, then EMP = 1 - PC.

This formula was, at least initially, applied to data obtained in this chapter for estimation of the P.Ph. pathway.

2. The heterotrophic synthesis of mannitol.

Touster and Shaw (1962) have described three types of reaction in which polyols may participate:

(a) polyol + NAD(P) $^{+}$ $\underline{\text{dehydrogenase}}$ keto-sugar + NAD(P)H

(b) polyol + NAD(P) $^{+}$ $\underline{\text{dehydrogenase}}$ aldo-sugar + NAD(P)H

These reactions may also involve ATP to yield sugar phosphates.

(c) polyol + ATP $\underset{\text{phosphokinase}}{\overset{\text{polyol kinase}}{\rightleftharpoons}}$ polyol-P + ADP

The first of these mechanisms has been suggested by Quillet (1957) to account for mannitol synthesis in Laminaria flexicaulis by the following pathway:

laminarin $\overset{\text{hydrolysis}}{\rightleftharpoons}$ glucose $\rightleftharpoons$ fructose $\overset{\text{reduction}}{\rightleftharpoons}$ mannitol

This is consistent with the finding of trace amounts of glucose and fructose in F. vesiculosus (Lindberg, 1953) and P. canaliculata (Lindberg and Paju, 1954).

The coenzyme to which the mannitol dehydrogenase enzyme is linked is not known in this instance; in Acetobacter suboxydans a particulate enzyme has been extracted with the requirements for either or no added cofactor.

Yamaguchi et al. (1966) have investigated the photosynthetic incorporation of $^{14}CO_2$ into mannitol in brown algae. Whilst evidence from the assay of enzyme activities is generally in accord with a dehydrogenase mediated reaction from hexose or hexose-P to mannitol, an interesting anomaly is indicated. By the preparation of dimedon derivatives of labelled mannitol they have shown the ratio of label in ^{14}C total/$^{14}C_1$ + $^{14}C_6$ to decrease with time from 8.0 to 3.0. This indicates that in mannitol synthesis C-2 to 5 are labelled earlier than C-1 and C-6, and suggests the possibility of synthesis from a triose precursor. Although mannitol synthesis from such precursors has been demonstrated in yeasts (Onishi and Suzuki, 1968) there is no direct evidence that such a system might be operative in algae.

Experimental

1. The respiration of exogenously supplied glucose-1-, -6-, and -U-^{14}C.

Apical and basal tissue of F. spiralis and Pelvetia was incubated in darkness in 3ml. sea-water media containing 2μCi of glucose-1- or -6-^{14}C in hermetically sealed 25ml. conical flasks. Each was shaken for 2-20 hours at 10°C. In addition, a third incubation contained 2μCi glucose-U-^{14}C; the data obtained from these incubations was not required for the estimation of the P.Ph. pathway by the method of Katz and Wood, but was used in a modified method to be described later.

$^{14}CO_2$ released in respiration of the glucose substrates was collected in 1ml. of 10% KOH contained in small phials standing in the flasks.

The activity of utilized glucose, equivalent to the depletion of initial activity in the medium, was estimated as $^{14}CO_2$ plus alcohol soluble and insoluble fractions.

Because of differences in specific activities of the isotopes used, all data were corrected accordingly.

glucose-1-^{14}C = 2.9μCi/M; 2μCi = 0.69μM.

glucose-6-^{14}C = 3.4μCi/M; 2μCi = 0.59μM.

2μCi was added to each incubation, and thus whilst the activity supplied was equal the amount of glucose was not.

The glucose-6-^{14}C contained 3.4/2.9 times more radioactivity than the glucose-1-^{14}C, per µM added. Results for glucose-1-^{14}C were thus multiplied by this factor.

Correction was also made for different weights of glucose substrates added. The final corrections applied were thus as follows:

glucose-1-^{14}C x 3.4/2.9 x 1/0.69

glucose-6-^{14}C x 1/0.59

Results shown in table 6:1 are expressed as cpm/mg. dry wt./µM.

Most apparent from these results is the discrepancy in calculated values for the P.Ph. pathway determined for short and long periods of incubation (1 vs. 2, and 4 vs. 5 in table 6:1). Were this difference due to recycling of hexose-P formed in the P.Ph. pathway, then calculated values should diminish with time. In theory, the values, having been corrected for recycling should have remained constant, but in practice this was obviously not so.

Barbour, Buhler and Wang (1958) and Katz and Wood (1963), used minimum incubation times of 20 hours. The uptake patterns established in brown algae by Drew (1969) and Jackson (unpublished data) showed that in incubations of this length the specific activity of $^{14}CO_2$ released in respiration increases latterly at a proportionally greater rate than the specific activity of other compounds (alcohol soluble + insoluble

Table 6:1. Calculation of the pentose-phosphate pathway by the method of Katz and Wood.

Tissue	Time (hours)	Isotope	$^{14}CO_2$ evolved	glucose utilized	% P.Ph. pathway
Fucus apices	2	gl-1-^{14}C	34.8	1225	0.85
		gl-6-^{14}C	4.6	1305	
Fucus apices	15	"	269.0	2151	8.7
			69.0	2079	
Fucus bases	2	"	136.5	1697	2.7
			7.3	1754	
Pelvetia apices	2	"	69.5	2431	0.87
			7.8	2713	
Pelvetia apices	20	"	4580.0	5522	50.5
			748.0	2428	
Pelvetia bases	2	"	82.4	2362	1.1
			6.1	2148	

Note: (1) radioactive data are expressed as cpm/mg.dry wt./µM, and are corrected to values such that all ^{14}C-atoms have the same specific activity as C-6 from glucose-6-^{14}C.

fractions) which become labelled, and which in toto ($^{14}CO_2$ + alcohol soluble and insoluble fractions) represent "depletion of media" used in calculating the specific yields of Katz and Wood. This is reflected also in increased oxidation-assimilation ratios with time. It is this initial lag in $^{14}CO_2$ released in respiration during the early stages of incubation which primarily make the Katz and Wood formula inappropriate for either long or short term estimations of the P.Ph. pathway. Cheldelin, Wang and co-workers (see Cheldelin, 1961) have partly resolved this problem by the use of a relative time unit, the time taken for the attainment of isotopic steady state, but this suffers in the time of its achievement (several hours) in which time recycling could be assumed to have taken place. Since their formula is based on a model in which no recycling is implicit, their method, too, becomes inappropriate.

It is thus apparent, when using short term incubations (which most nearly meet the theoretical ideal for the calculation of pathways), that the use of specific yields produces spurious results due to isotopic non-steady state conditions. Absolute CO_2 yields do not share this disadvantage as no reference is made to other metabolites.

As mentioned previously, simultaneous incubations were carried out using glucose-U-^{14}C substrate. The results of

these incubations allow comparison to be made between $^{14}CO_2$ released from C-1 and C-6 with that from the other C-atoms of the uniformly labelled molecule. It is here assumed that glucose-U-^{14}C is not only labelled at each carbon atom but that each of these atoms has equal specific activity (Radiochemicals Ltd., Amersham, personal communication).

Corrections were applied to glucose-1- and -6-^{14}C as before. Corrections to data from glucose-U-^{14}C incubations were made as follows. Assuming, for example, that glucose-6- and -U-^{14}C as supplied had equal specific activities per molecule, then C-6 of glucose-6-^{14}C would contain 6 times more radioactivity than any carbon atom of glucose-U-^{14}C. However, as supplied, these isotopes had activities of 3.4 and 3.1mCi/mM respectively. Thus C-6 of glucose-6-^{14}C was $6 \times 3.4/3.1 = 6.6$ times more radioactive than any carbon atom of glucose-U-^{14}C, and this correction factor was applied for the data in table 6:2. Hence, corrections have been made so that all labelled carbon atoms can be considered to have the same specific activity as C-6 from glucose-6-^{14}C.

2. Recalculation of the P.Ph. pathway contribution.

The data shown in table 6:2 indicate not only the $^{14}CO_2$ released from C-1 and C-6 but also that $^{14}CO_2$ liberated from the uniformly labelled molecule. Hence, by difference, the contribution of C-2 to 5 can be estimated.

In metabolism via the EMP pathway followed by oxidation of pyruvate, ^{14}C from the hexose carbons appears in CO_2 released in the following order: $3 = 4 > 2 = 5 > 1 = 6$. Therefore, when the EMP pathway is predominant over the P.Ph. pathway, $^{14}CO_2$ from glucose-U-^{14}C should be considerably greater than that from glucose-1- or -6-^{14}C due to the earlier contribution of C-2 to 5 to respiratory CO_2. In the P.Ph. pathway C-1 is converted solely to CO_2 and this conversion is rapid as compared with the subsequent loss of C-2 to 6 (by randomization due to recycling of hexose-P) in the same pathway.

In table 6:2 it is apparent that, in apical tissue of Fucus for example, C-1 liberated as CO_2 from glucose-1-^{14}C accounted for virtually all the activity from the uniformly labelled molecule. Assuming that all glucose molecules were metabolized identically, regardless of positional labelling differences, this indicates that C-2 to 6 are involved only to a slight extent in CO_2 production. This provides evidence for an active P.Ph. pathway in this species, and suggests this activity to exceed that value (0.85%) calculated by the method of Katz and Wood.

These data can be used further to yield quantitative data as follows. Consider, for example, data for Pelvetia apical tissue (table 6:2). After 2 hours incubation 142 cpm/mg. dry wt./µM had been liberated in $^{14}CO_2$ from glucose-U-

Table 6:2. Re-calculation of the pentose-phosphate pathway using $^{14}CO_2$ yields from glucose-1-, -6-, and -U-^{14}C.

Tissue	Time (hours)	Isotope	$^{14}CO_2$ evolved	% P.Ph. pathway Katz & Wood	% P.Ph. pathway Jackson
Fucus apices	2	gl-1-^{14}C	34.8	0.85	76.5
		gl-6-^{14}C	4.6		
		gl-U-^{14}C	39.6		
Fucus apices	15	"	269.0	8.7	74.0
			69.0		
			270.0		
Fucus bases	2	"	136.5	2.7	10.4
			7.3		
			1242.0		

Table 6:2. (Continued)

Tissue	Time (hours)	Isotope	$^{14}CO_2$ evolved	% P.Ph. pathway Katz & Wood	% P.Ph. pathway Jackson
Pelvetia apices	2	gl-1-^{14}C	69.5	0.87	43.5
		gl-6-^{14}C	7.8		
		gl-U-^{14}C	142.0		
Pelvetia apices	20	"	4580.0	50.5	39.6
			748.0		
			9660.0		
Pelvetia bases	2	"	82.4	1.1	46.5
			6.1		
			164.0		

Note: (1) data for glucose-1- and -6-^{14}C are from table 6:1.

(2) cpm/mg. dry wt./µM data are corrected to values such that all C-atoms have the same specific activity as C-6 from glucose-6-^{14}C.

^{14}C. Of these counts, 69.5 were accounted for by ^{14}C-1 (from glucose-1-^{14}C), and 7.8 were accounted for by ^{14}C-6 (from glucose-6-^{14}C). As C-1 and C-6 are equivalent in the EMP pathway, then 69.5 - 7.8 = 61.7 cpm/mg. dry wt./µM were released at the 6-phosphogluconate cleavage in the P.Ph. pathway (assuming the 7.8 cpm to have been released via the EMP pathway). Thus, of 142 cpm liberated as $^{14}CO_2$, 61.7 cpm are accounted for by the P.Ph. pathway, which represents 61.7/142 x 100 = 43.5%. Expressed algebraically, this is equivalent to:

$$Gp = \frac{(G_1 - G_6)}{G_U} \times 100\%$$

Gp = the proportion of glucose metabolism proceeding via the P.Ph. pathway.

G_1, G_6, G_U = corrected $^{14}CO_2$ yields (cpm/mg. dry wt./µM) from glucose-1-, -6-, and -U-^{14}C respectively.

If the P.Ph. pathway and the EMP pathway are assumed to operate exclusively, then EMP is calculated by difference. Evidence that this might not be so, at least in vitro, is presented in the next chapter.

3. The heterotrophic synthesis of mannitol.

In chapter 4 mannitol was shown to be the major product of the heterotrophic uptake of glucose in Pelvetia,

as was previously demonstrated by Bidwell and Ghosh (1963) and Drew (1969).

Using data obtained in the previous section, in which asymmetrically labelled glucose substrates were offered to Pelvetia, information can be obtained concerning the distribution of label in heterotrophically formed mannitol. This approach removes the need to prepare dimedon derivatives, but assumes that all glucose molecules regardless of different labelling patterns produce equal amounts of mannitol, and that mannitol is the only quantitatively significant soluble product (i.e. ethanol soluble fraction ≃ mannitol produced).

Corrections for different specific activity were made to data for the ethanol soluble fraction as before. Data for Pelvetia apical and basal tissues are presented in table 6:3.

The result for Pelvetia basal tissue is consistent with symmetrically labelled mannitol derived from hexose conversion. The value for apical tissue, however, is suggestive of early terminal carbon labelling; mechanisms whereby this might occur are obscure, and no explanation can be offered here.

Table 6:3. Labelling patterns in heterotrophically synthesized mannitol from Pelvetia after 2 hours incubation in glucose-1-, -6-, and -U-^{14}C.

Tissue	Isotope	EtOH sol. (~ mannitol)	$\frac{^{14}C \text{ total}}{^{14}C_1 + {}^{14}C_6}$
Pelvetia base	gl-1-^{14}C	1850	3.08
	gl-6-^{14}C	1560	
	gl-U-^{14}C	10500	
Pelvetia apex	"	2060	0.9
		2370	
		3940	

CHAPTER 7

Enzymic particulate preparations of F. spiralis and Pelvetia.

Contents

Introduction

Although a considerable literature has amassed concerning the occurrence of carbohydrates in marine algae, little is known of the enzymes which mediate their metabolism. The extraction of functional enzymes from Phaeophyta has proved to be difficult. Ohmann (1963) found transaminase and dehydrogenase activity in Dictyota dichotoma and Ectocarpus sp., but not in extracts of Cystoseira mediterranea, which inhibited the authentic enzymes, as did extracts of Fucus and Laminaria (Jacobi, 1958). Yamaguchi et al. (1966) found low or negligible enzyme activities in Eisenia bicyclis, but high activities, particularly of mannitol-1-phosphatase, in D. dichotoma and Spathoglossum pacificum.

The problem of low enzyme activity in extracts of brown algae has been partly resolved by Lin and Hassid (1966)

and Johnston and Davies (1969). These workers incorporated polyvinylpyrollidone (PVP) in their extraction media; PVP has been postulated to form complexes with inhibitory phenolic compounds released during the extraction procedure (Johnston and Davies, 1969), thus preventing their inhibitory effect.

Using a particulate fraction obtained from a PVP treated homogenate of F. gardneri, Lin and Hassid (1966) have demonstrated several enzymes of the glycolytic pathway and TCA cycle in Fucus and Laminaria. The operation of the cycle is evidenced by the detection of several intermediates (Jacobi, 1958, 1962). Some tracer evidence for the TCA cycle is also provided by the results of Bidwell and Ghosh (1963). Johnston and Davies (1969) conclude that despite this evidence the presence and interconversion of enzymes and intermediates can be accounted for without the operation of the complete cycle, or the involvement of the glyoxylate cycle, for which no evidence was in fact found (no isocitrate lyase activity was evident). Bidwell and Ghosh (1963) did, however, demonstrate the labelling of malate prior to succinate when formate-^{14}C was offered to intact thalli of F. vesiculosus, a feature consistent with the operation of the glyoxylate cycle in this species.

Johnston and Davies also demonstrated glucose-6-P

dehydrogenase, particularly active in extracts of Laminaria. This indicates a possible means of hexose-pentose interconversions or the involvement of the P.Ph. pathway. Although not previously identified in brown algae, this and other enzymes of the P.Ph. pathway have been detected in blue-green algae (Richter, 1959) and green algae (Jacobi, 1957), but are believed to be absent from many red algae (Jacobi, 1957).

There is no evidence for the operation of complete respiratory pathways or metabolic sequences using a single precursor. In this chapter preliminary experiments were carried out in order to investigate the possibility of mannitol synthesis from glucose by particulate preparations of Pelvetia, and further, to see if coenzyme levels could be so adjusted to effect a similar conversion in Fucus. From chapter 6 it is apparent that respiration in Fucus apical tissue must largely produce NADPH, whilst in Pelvetia and Fucus basal tissue both coenzymes will be formed.

It is appreciated that any pH selected for the extraction or incubation media must be a compromise for the activity of the various enzymes, which if present would mediate the reaction sequence.

Experimental

1. Materials and methods.

The extraction medium used in these experiments was a modification of that used by Lin and Hassid (1966), and included 0.5% bovine serum albumen (BSA) and 3×10^{-4}M mercaptoethanol.

600 ml. of the extraction medium were made up at pH 9.0 as follows:

sucrose	0.4M
tris (hydroxymethyl) amino methane	0.2M
EDTA	5mM
monobasic potassium phosphate	0.01M
sodium citrate	0.02M
bovine serum albumen (BSA)	0.5%
mercaptoethanol	3×10^{-4}M
polyvinylpyrrolidone (PVP)	5%

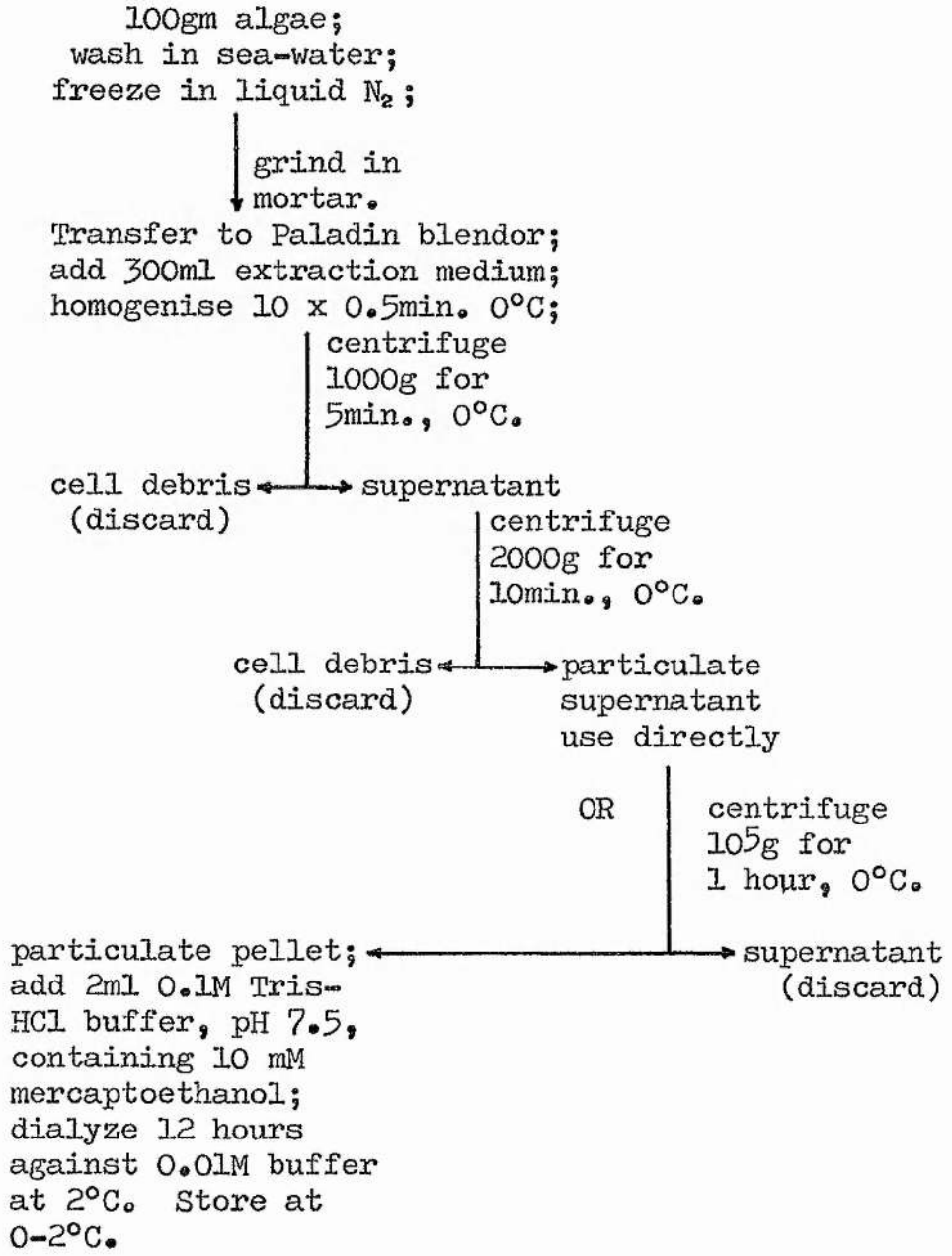
2. Preliminary experiments.

Three preliminary experiments were carried out using algal extracts prepared as shown in the synopsis.

Experiment (a)

If mannitol is formed heterotrophically by the direct reduction of hexose (or hexose-P) it is reasonable to assume that in the presence of the correct co-enzyme the reduction will take place without complete respiratory function.

Synopsis of extraction procedure.



Sodium citrate was included only in the first experiment in which it was required to block the EMP pathway. Citrate in the presence of ATP is inhibitory to phosphofructokinase activity at concentrations exceeding 0.2mM, by second site allosteric effect (Garland et al., 1963 ; Parmeggiani and Bowman, 1963). 20mM were present in the extraction medium of Lin and Hassid (1966).

NADPH was added to inhibit, at least temporarily, the P.Ph. pathway in those incubations notated +, but was omitted from those designated -. $NADP^+$ was not included in any of the incubations. Reaction mixtures were made up in 10ml. conical flasks as follows:

ATP.....50µmole

$MgCl_2 . 6H_2O$.....10µmole

0.1M tris-HCl buffer containing

10mM mercaptoethanol (pH 7.5)..... 0.5 ml.

particulate preparation..... 1.0 ml.

glucose-U-^{14}C (5µCi)..... 0.5 ml.(= 325 µmole)

NADPH (+ series only)..... 0.25mM

A small phial containing 0.5ml. 10% KOH was added to the flask, which was then hermetically sealed, and incubations were carried out at 10°C for 20 hours. 0.5 ml. aliquots of the reaction mixture were withdrawn after 1, 5, and 20 hours, and were killed by addition of 0.5ml. boiling 80% ethanol.

The coagulated particulate fraction was kept at 60°C for 15 minutes to ensure extraction, and was then centrifuged. The pellet was found to contain less than 5% of the total radioactivity. The supernatant fraction was analysed by paper chromatography. Solvent systems used for chromatography were ethyl methyl ketone, glacial acetic acid, saturated boric acid (EMK. 9:1:1) and iso-propanol, n-butanol, water (IPrBu. 14:2:4).

$^{14}CO_2$ estimated as $Ba^{14}CO_3$ at the termination of the incubations. This procedure was as described previously.

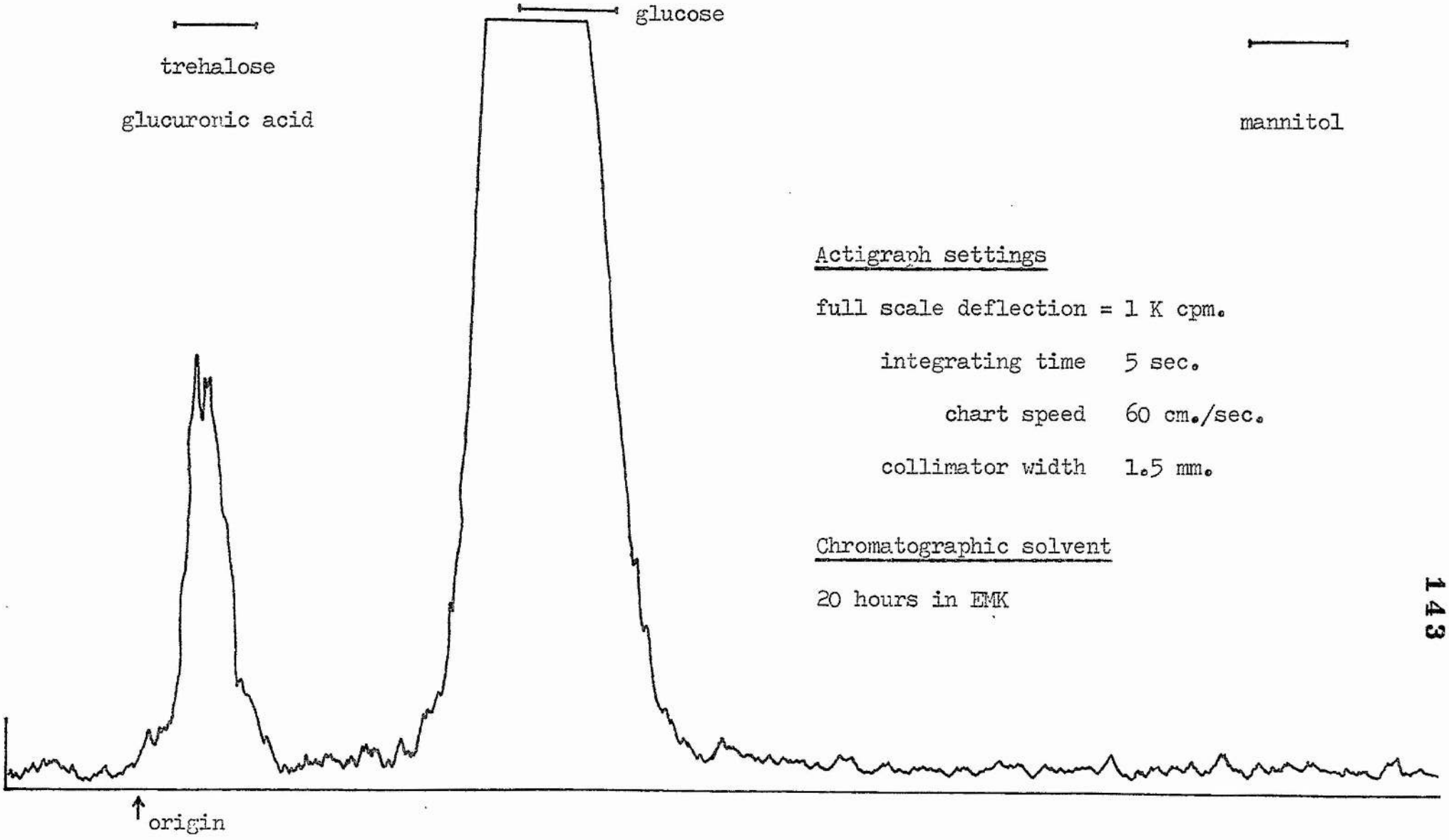
The results are shown in table 7:1, and Actigraph scans of the distribution of label are shown in figures 7:1 - 7:3.

These results indicate that particulate preparations are capable of respiration, at least so far as to effect $^{14}CO_2$ release. Higher rates of respiration (under these conditions) are evident in Fucus, possibly a consequence of respiration via the P.Ph pathway. Contrary to expectation the Fucus + incubation was more active than Fucus -, possibly due to a requirement for the reduced coenzyme itself or to its oxidation and subsequent enhancement of the P.Ph. pathway. However, no peaks were evident at 260 or 340 nm, the absorbtion maxima of oxidised and reduced NADP. The fate of the coenzyme could not, therefore, be ascertained spectrophotometrically at the concentrations present. More than 90%

Table 7:1. Composition of incubation media and respiration of particulate preparations of *F. spiralis* and *Pelvetia*.

	NADPH	$NADP^+$ (mM)	NADH	NAD^+	citrate	ATP	ADP (µmole)	$^{14}CO_2$/20 hr.
Fucus +	0.25	-	-	-	+	50	-	224
Fucus -	-	-	-	-	+	50	-	128
Pelvetia +	0.25	-	-	-	+	50	-	72
Pelvetia -	-	-	-	-	+	50	-	89

Figure 7:1. Distribution of label in the alcohol soluble fraction of Fucus particulate preparation incubated in the presence or absence of NADPH.



Actigraph settings

full scale deflection = 1 K cpm.

integrating time 5 sec.

chart speed 60 cm./sec.

collimator width 1.5 mm.

Chromatographic solvent

20 hours in EMK

Figure 7:2. Distribution of label in the alcohol soluble fraction of Pelvetia particulate preparation incubated in the presence or absence of NADPH

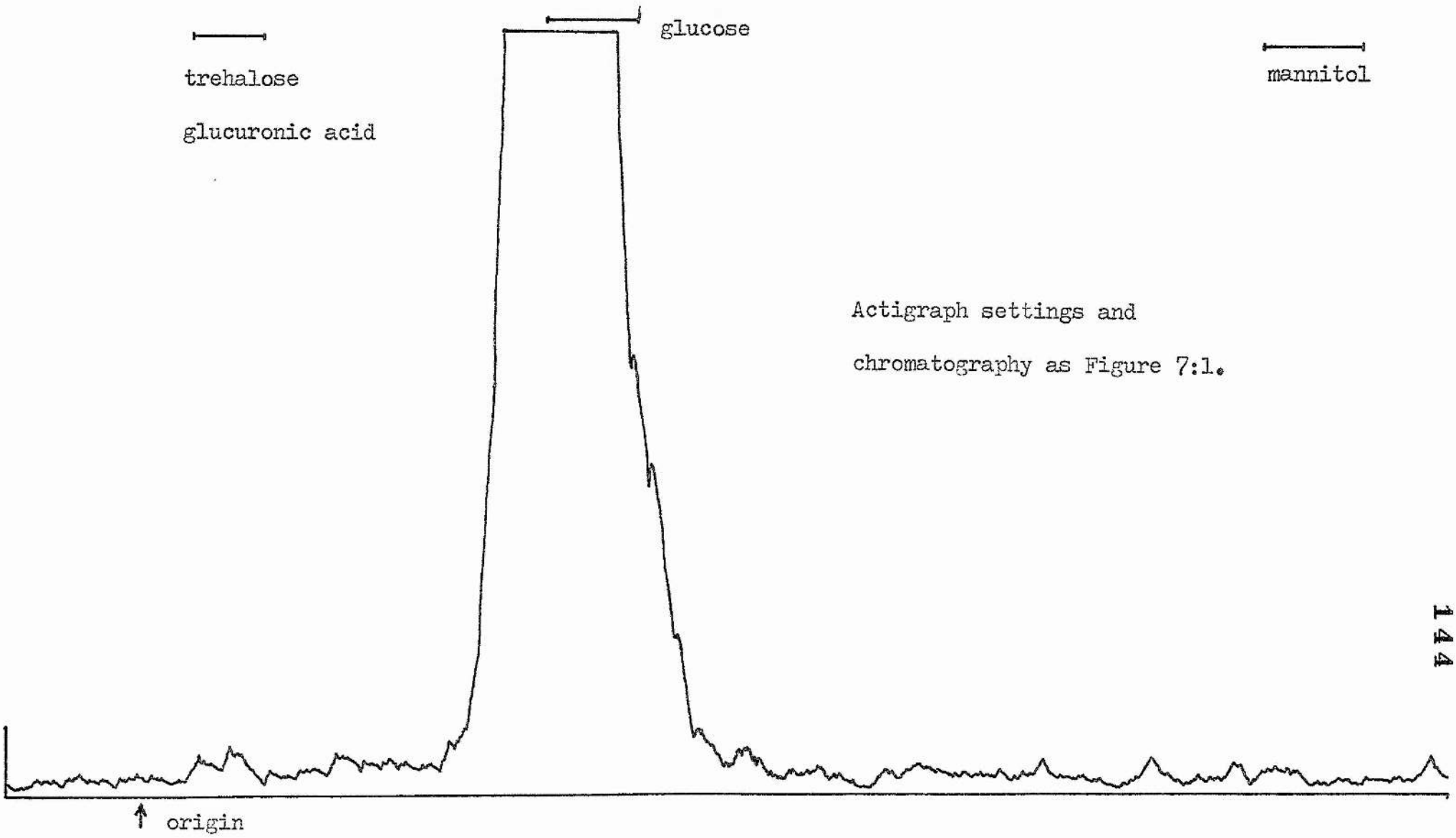
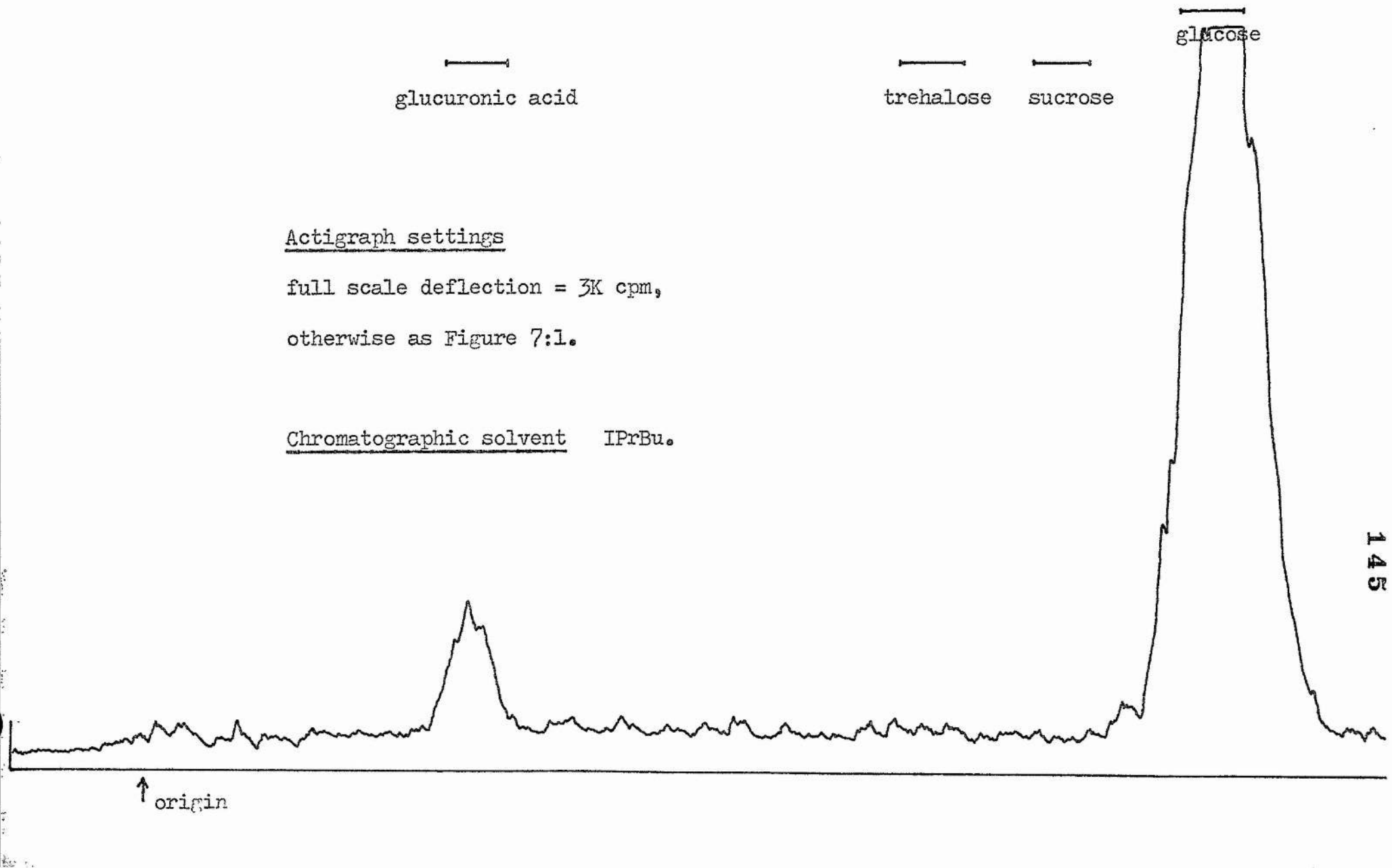


Figure 7:3. Distribution of label in the alcohol soluble fraction of Fucus particulate preparation incubated in the presence or absence of NADPH.



absorbance was evident at 272nm, possibly due to poly-phenols (free or PVP-bound), or to amino acids such as tyrosine or phenylalanine.

Paper chromatographic analysis showed the presence of a single peak (other than glucose) in Fucus, and only traces of the same peak in Pelvetia (figures 7:1 -7:2). This peak, which had a mobility identical with trehalose in the EMK solvent, was not susceptible to acid hydrolysis, trehalase or acid phosphatase treatment. Identical mobility with free glucuronic acid was later demonstrated in the IPrBu and EMK solvents. Chemical identity was not established beyond this. Maximal labelling of the compound was observed after 1 hour; thereafter the extent of labelling remained more or less constant.

Experiment (b).

This experiment was conducted essentially as before except that ADP and $NADP^+$ were included so that initial ADP/ATP and $NADP^+$/NADPH ratios exceeded unity.

It was expected that under these conditions respiration would be enhanced in both species, and that if the EMP pathway were effective or if NADPH were converted to NADH, then mannitol might at least be synthesized in Pelvetia. This, from knowledge of the respiratory pathways in vivo, assumes NADH to be the hydrogen donor for the synthesis of mannitol.

The results are shown in table 7:2, and Actigraph scans

Table 7:2. Composition of incubation media and respiration of particulate preparations of F. spiralis and Pelvetia.

	NADPH	$NADP^+$ (mM)	NADH	NAD^+	citrate	ATP (µmole)	ADP	$^{14}CO_2$/20 hr.
Fucus +	0.25	1.0	-	-	-	16	30	778
Fucus -	-	-	-	-	-	16	30	364
Pelvetia +	0.25	1.0	-	-	-	16	30	2960
Pelvetia -	-	-	-	-	-	16	30	604

of the distribution of label are shown in figures 7:4 and 7:5.

The removal of citrate and the addition of ADP and $NADP^+$ has obviously caused increased respiration in this experiment, particularly in Pelvetia.

In all cases free glucuronic acid was detected. Other, low mobility compounds were not identified. No mannitol was evident from any of the incubations.

Experiment (c)

This experiment was conducted as before, but NAD was included in incubation media instead of NADP. From table 7:3 it is evident that in all cases respiration was diminished with respect to expt. (b).

Regardless of these differences, labelling patterns were identical with those found in the second experiment, again with no mannitol synthesis.

These experiments idicate the necessity for much more work on particulate preparations, with particular regard for enzyme assay. Some metabolic activity is evidenced by $^{14}CO_2$ release, suggesting that the respiratory pathways may be at least functional, but the pathway of mannitol synthesis appears to have been destroyed in the extraction process.

Table 7:3. Composition of incubation media and respiration of particulate preparations of *F. spiralis* and *Pelvetia*.

	NADPH	$NADP^+$ (mM)	NADH	NAD^+	citrate	ATP (µmole)	ADP	$^{14}CO_2$/20 hr.
Fucus +	-	-	0.25	1.0	-	16	30	153
Fucus -	-	-	-	-	-	16	30	321
Pelvetia +	-	-	0.25	1.0	-	16	30	205
Pelvetia -	-	-	-	-	-	16	30	139

Figure 7:4. Distribution of label in the lcohol soluble fraction of Fucus particulate preparation incubated in the presence of $NADP^+$

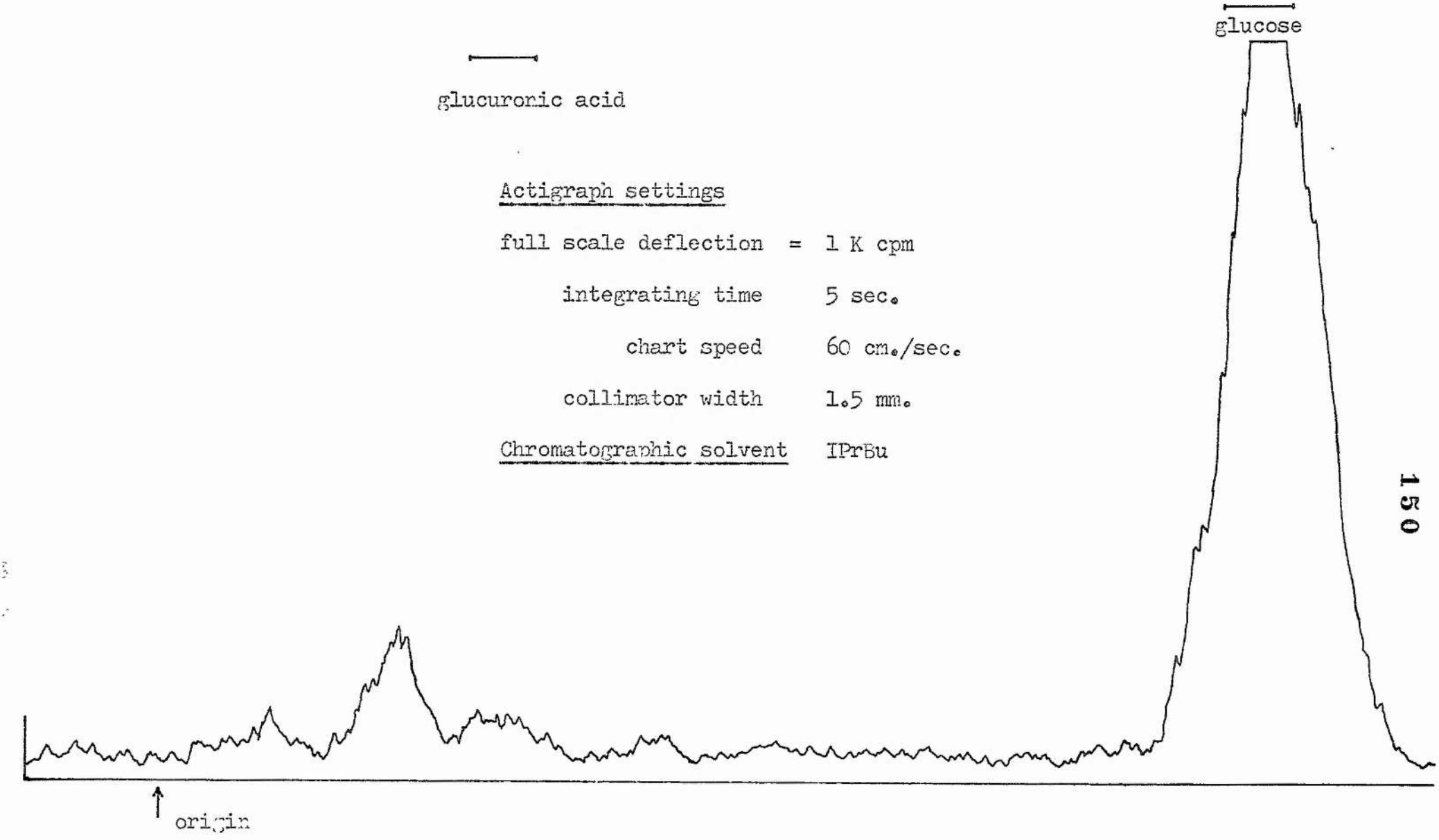
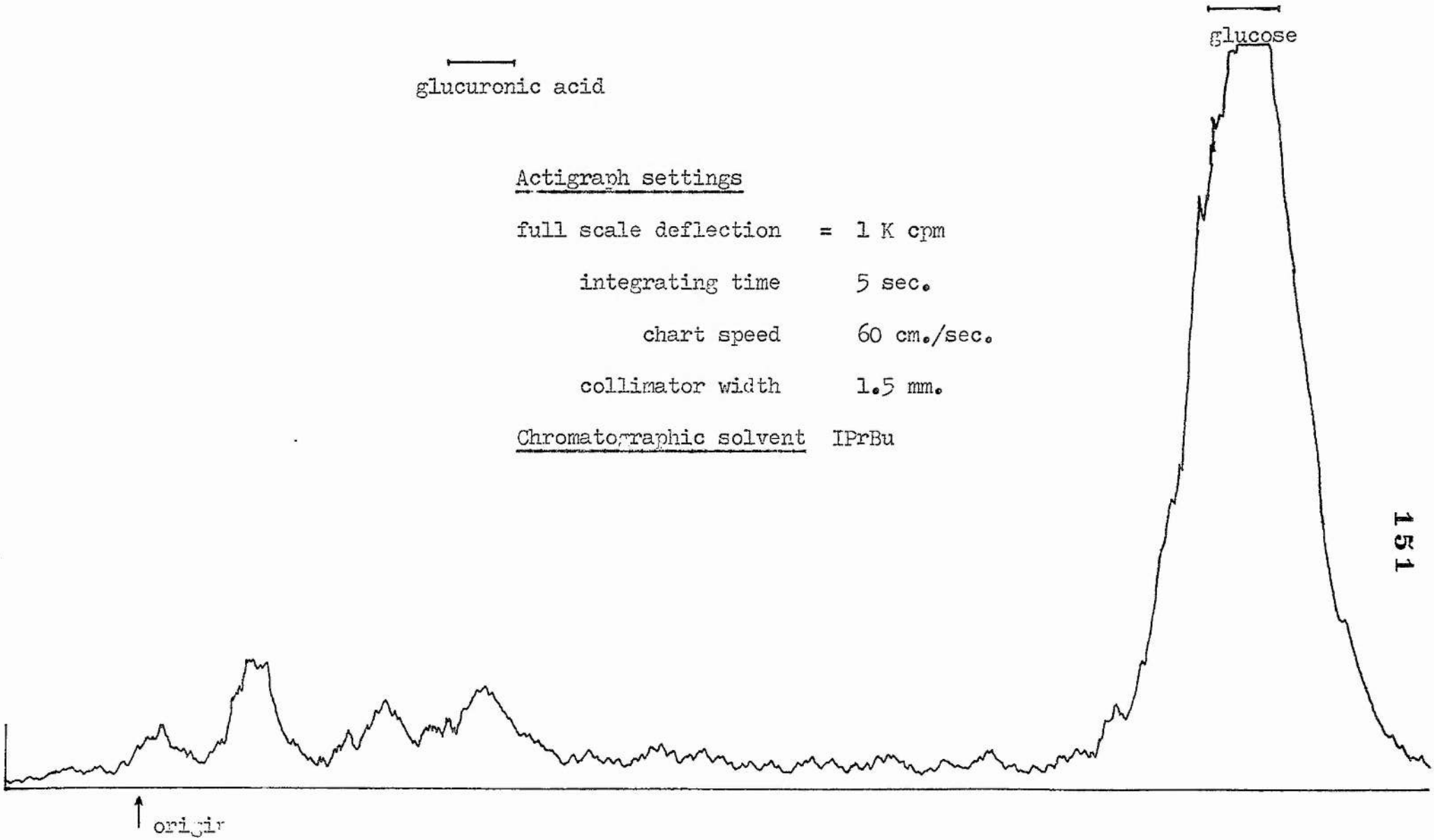


Figure 7:5. Distribution of label in the alcohol soluble fraction of Pelvetia particulate preparation incubated in the presence of $NADP^+$.



CHAPTER 8

The efficiency of heterotrophy as a means of carbon accretion as compared with dark fixation, photoassimilation and photosynthesis.

Contents page

Introduction

Heterotrophic uptake of organic substrates in the Phaeophyceae has been treated semi-quantitatively by Bidwell and Ghosh (1963) and Drew (1969). Whether such accretion could offset endogenous respiratory carbon losses remains extremely dubious.

Dark, or heterotrophic fixation of CO_2 has been examined extensively in the photosynthetic tissues of succulent and non-succulent land plants (Thurlow and Bonner, 1948; Stutz and Burris, 1951; Saltman et al., 1957; Joshi et al., 1962) and in various aquatic plants (Calvin et al., 1951; Rho, 1959; Joshi et al., 1961, 1962; Craigie, 1962). In terrestrial and fresh water plants label from $^{14}CO_2$ appears predominantly in organic acids, particularly malate. Marine plants tend to form amino acids much more readily. Rho (1959) has demonstrated the production of ornithine, citrulline, histidine and lysine during

dark fixation by the marine diatom Nitzschia closterium. Joshi et al. (1961) showed that 80 - 90% of the activity was in the amino acid fraction in Zostera marina (angiosperm), Egregia laevigata (brown alga) and Gigartina canaliculata (red alga). Furthermore, they demonstrated that in saline media cell-free preparations of spinach leaves showed this marine type of dark fixation. Craigie (1962) demonstrated the production of amino acids in Fucus vesiculosus and Polysiphonia lanosa, although the distribution of label amongst the products was different from that demonstrated in Egregia by Jacobi et al., (1962). The important point concerning dark fixation of CO_2, however, is that the carboxylation reactions which must ensue for synthesis to take place, are endergonic, requiring expenditure of energy by the cells. Thus this process cannot be considered when estimating the heterotrophic potential of a plant.

Qualitative and quantitative aspects of algal photosynthesis have received considerable attention from several authors. Bidwell and collaborators have considered Phaeophycean photosynthesis extensively (Bidwell, 1958, 1967; Bidwell et al., 1958). Carbon balance figures for F. vesiculosus have been presented by Sieburth (1969b).

Very little data exists on the subject of photoassimilation. Nalewajko et al. (1963) showed heterotrophic uptake in Chlorella to be enhanced at low light intensity, whilst Bunt (1969)

ascertained that in the marine diatom Cocconeis (species not identified) heterotrophic uptake of exogenous organic metabolites takes place only in the light.

It was decided that the various modes of carbon accumulation should be considered in order to ascertain the relative importance of these methods and to determine the effect, if any, of exogenously supplied glucose on the process of photosynthesis. Pelvetia was chosen as the subject material as this species is the most efficient algal heterotroph that has been investigated.

Experimental

Excised apices of Pelvetia were incubated at 10°C in 10 ml. sea water media for 2 hours in conditions of darkness, low light intensity (1.43×10^{-3} cal/min/cm.^{2}) and high light intensity (0.245 cal/min/cm.^{2}), and in the presence of exogenously supplied glucose-^{12}C (140µgC), glucose-U-^{14}C (6µCi ≡ 140µg C) and $H^{14}CO_3^-$ (10µCi) as indicated in table 8:1. Details of analysis were as described previously.

The results are shown in table 8:1.

At low light intensity dark fixation and 'photosynthesis' (treatments 1 and 2) were essentially equivalent, at least quantitatively, and probably qualitatively also.

In the presence of exogenous glucose-^{12}C dark fixation was reduced (7). It is possible that this difference is not significant, or that data reflect a common metabolic step for which the two processes compete.

The processes of photoassimilation (5,6) and heterotrophy (4) appear to be identical, both quantitatively and qualitatively; in each case label was detected in mannitol. Photoassimilation was not stimulated by increasing light intensity, although a greater proportion of the label did appear in the insoluble fraction.

At high light intensity (3) photosynthesis proceeded at a rate 50 times that at low light intensity (2), where photochemical reactions were obviously limiting. The conversion

to insoluble products was high in absolute terms, but low when expressed as % total activity. This is due to the considerable amount of activity directed to the mannitol storage pool.

An exceptionally interesting feature was the enhancement of photosynthesis in the presence of exogenous glucose-^{12}C (8,9) as compared to the controls in which glucose was absent (2,3). Under conditions of high light intensity the enhancement was by 43%.

The significance of these data will receive attention in the general discussion which follows.

Table 8:1. The efficiency of heterotrophy as a means of carbon accretion in Pelvetia as compared with dark fixation, photoassimilation and photosynthesis.

	light intensity & C source	nominal mode of ^{14}C accretion	EtOH sol. (cpm/mg. dry wt.)	EtOH insol. (cpm/mg. dry wt.)	carbon fixed EtOH sol. (µg C/100 mg. dry wt./2 hr.)	carbon fixed EtOH insol. (µg C/100 mg. dry wt./2 hr.)	Total C fixed (µg C/100 mg. dry wt./2 hr.)
1.	dark; $H^{14}CO_3^-$	dark fixation	1085	213	13.05	2.56	15.61
2.	L.L.; $H^{14}CO_3^-$	photosynthesis dark fixation	1105	205	13.29	2.47	15.76
3.	H.L.; $H^{14}CO_3^-$	photosynthesis	61500	5200	740.00	63.00	803.00
4.	dark; gl-U-^{14}C	heterotrophy	490	216	3.20	1.41	4.61
5.	L.L.; gl-U-^{14}C	photoassimilation heterotrophy	478	220	3.12	1.44	4.56
6.	H.L.; gl-U-^{14}C	photoassimilation	420	280	2.74	1.83	4.57
7.	dark; $H^{14}CO_3^-$ + gl-^{12}C	dark fixation	960	232	11.55	2.79	14.34
8.	L.L.; $H^{14}CO_3^-$ + gl-^{12}C	photosynthesis dark fixation	1230	199	14.80	2.39	17.19
9.	H.L.; $H^{14}CO_3^-$ + gl-^{12}C	photosynthesis	91000	4000	1095.00	48.00	1143.00

Note: (1) L.L. and H.L. are low and high light intensities respectively.

CHAPTER 9

General Discussion

Contents

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1. Carbohydrate content and the physiological role of the polyols.

(a) Carbohydrate content of the tissues.

Chromatographic analysis of ethanolic extracts of both Pelvetia and F. spiralis failed to reveal significant amounts of free sugars. However, trace amounts of a compound having chromatographic retention close to that of fructose were found in apical tissue of Pelvetia. A second unknown, found in Pelvetia, F. spiralis and F. serratus, possibly corresponds to mannitol acetate or laminitol. These results are in accord with those of Lindberg and co-workers (1953-1954).

Quantitation of polyols has shown remarkably similar total contents consisting solely of mannitol in Fucus, and both mannitol and volemitol in Pelvetia. This equality is evident both in winter and summer, except in basal tissue of F. spiralis, which exhibits a winter mannitol maximum.

Distribution patterns of mannitol and volemitol content along the thallus exhibit sub-apical maxima in vegetative thalli of Pelvetia. A similar pattern for mannitol content

was shown in F. vesiculosus (Moss, 1950) at the onset of reproductive activity, but in that work vegetative thalli showed a negative gradient from apex to base.

(b) Differences in carbohydrate metabolism in intact and excised thalli.

The regional differences in endogenous polyol content described above and the observation that the regions exhibit different abilities to take up exogenously supplied glucose (Chapter 4), suggested the possibility of carbohydrate translocation in both species. However, data in figures 5:1 - 5:3 show no direct evidence for translocation of significant amount of carbohydrate. The interesting feature of these experiments, however, was the increase in uptake, $^{14}CO_2$ released and the specific activity of that $^{14}CO_2$ (table 9:1) as compared to data shown for the corresponding excised tissues in table 4:1. This was particularly evident in the experiment in which the apical tissue of F. spiralis was immersed, when uptake and $^{14}CO_2$ recovery were increased more than one hundred-fold over the corresponding values for excised apical tissue. Had glucose uptake into the immersed portion of thallus been similar in excised and intact tissue and had the specific activity of $^{14}CO_2$ released in respiration been higher in the latter tissue it may have

been possible to postulate the mass translocation of an immediately respirable substrate to emersed portions of the thallus. Since the enhancement factors for uptake, $^{14}CO_2$ recovery and the specific activity of the $^{14}CO_2$ were similar then it is not possible to make such an hypothesis. The observed enhancement must be attributed to an effect on the immersed portion of thallus *per se*. Tissue integrity thus seems to be an important factor in glucose uptake and metabolism.

The enhancement values for *basal tissue* of intact thalli of *F. spiralis* and *Pelvetia* were much smaller. However, in each case the enhancement for $^{14}CO_2$ released was somewhat higher than that for total uptake or for the specific activity of the $^{14}CO_2$.

These observations can be accounted for as follows;

(i) There may be translocation of an immediately respirable substrate, such that $^{14}CO_2$ is released not only by the immersed but also the emersed tissue.

(ii) Assuming the initial endogenous pools in intact and excised thalli to be the same, then the relatively greater increase in $^{14}CO_2$ release from intact basal portions of thalli as compared with the increase in glucose-U-^{14}C uptake (table 9:1) cannot be accounted for solely in terms of increased specific activity of these pools. Yamaguchi *et al.* (1966) have demonstrated $^{14}CO_2$ released from *Eisenia bicyclis*

(containing photosynthetically labelled mannitol as the respiratory substrate) to be proportionately greater than expected value. They have proposed that newly formed substrate is more susceptible to respiratory oxidation than that substrate already present; a similar explanation will account for the relatively greater increase in $^{14}CO_2$ release as compared with total uptake in the intact basal tissue of Pelvetia (table 9:1). In effect, this suggests an increased oxidation:assimilation ratio for the exogenously supplied metabolite. The same explanation will not suffice for F. spiralis, which does not produce mannitol heterotrophically.

The greater enhancement of $^{14}CO_2$ as compared with its specific activity may be accounted for as follows:

(iii) The non-parallel increase of $^{14}CO_2$ released and its specific activity possibly indicates the simultaneous respiration of an unlabelled endogenous metabolite in the immersed tissue. This, however, is contrary to explanation (ii); it seems more likely that this effect can be accounted for in terms of respiration of unlabelled mannitol by the emersed tissue, as was shown to take place in chapter 3.

Although more research is indicated along these lines it seems that the role of trumpet hyphae in conduction is extremely dubious in these algae. More elaborate experiments by Jupp (personal communication) provide fairly conclusive evidence that trumpet hyphae in Laminaria hyperborea are

Table 9:1. Enhancement factors for uptake and respiratory ^{14}C loss in intact tissues of Pelvetia and F. spiralis.

	total uptake	$^{14}CO_2$ released in respiration	specific activity of $^{14}CO_2$ released
F. spiralis apex	X 125	X 112	X 85
F. spiralis base	X 4.8	X 20	X 8.5
Pelvetia base	X 1.7	X 17	X 10

Note: (a) these factors are derived from values for intact tissue (table 5:1) divided by the corresponding values for excised tissue (table 4:1).

(b) total uptake consists of uptake into the alcohol soluble and insoluble fractions.

also ineffective in mass carbohydrate conduction.

(c) The physiological role of polyols.

The virtual ubiquity of polyols (particularly mannitol) in brown algae indicates an important physiological role. In chapter 3 it was shown on purely theoretical grounds that polyols in F. spiralis and Pelvetia are probably not major factors in endogenous osmo-regulation in these species. Similar results have been obtained for the facultative halophytic fungus Dendryphiella salina (Jackson, unpublished data). It thus appears that high levels of endogenous polyols in saline media are not themselves responsible for increased endogenous osmotic pressure, but are a consequence of it. Allaway and Jennings (in preparation), working with the same species, have provided data substantiating this conclusion.

What physiological role can be assigned to polyols in these algae? Starvation experiments with Pelvetia indicate mannitol to be the respiratory substrate in this alga, a finding in agreement with Yamaguchi et al. (1966) and Bidwell (1967), also working on brown algae. During starvation, the volemitol pool was not depleted, suggesting that this polyol is not a respiratory substrate and neither is it interconvertable with mannitol even when the latter is seriously depleted. What function volemitol may

have is not clear; as it does not appear to serve specifically in metabolism it may function in the transfer of hydrogen on co-enzymes as postulated by Touster and Shaw (1962), and therby be effective in the control of the various metabolic pathways.

2. The respiratory and carbohydrate metabolism of F. spiralis and Pelvetia.

(a) Respiratory carbon loss.

In chapter 3 it was shown that the respiratory loss of carbon from F. spiralis is somewhat higher than from Pelvetia, and that in both these species apical respiratory losses are approximately double those sustained by basal tissue.

Bidwell and Craigie (1962) demonstrated 25% suppression of respiration in F. vesiculosus during emersion. In the present study a suppression of 75% has been shown for Pelvetia.

The above differences, in both immersed and emersed respiratory rate may have considerable ecological significance for Pelvetia enabling the species to survive at the top of the littoral zone, where photosynthesis may proceed at maximum efficiency for only short periods each day.

(b) The effect of glucose concentration on respiration.

In the presence of exogenously supplied glucose at

concentrations between 40 and 4000µg glucose/ml (0.22 - 22.0mM) there was no evident increase in respiratory rate in Pelvetia. This has been taken to be indicative of hexokinase deficiency by Taylor (1950; 1960a,b) and by Jacobi (1957a, b). It is apparent from the results of heterotrophic uptake experiments that there is considerable utilization of exogenously supplied glucose by this species, and unless complete direct oxidation of this glucose takes place it seems likely that hexokinase, if sought, might be successfully demonstrated. Certainly some of the glucose appears to undergo direct oxidation, at least in Fucus, and conceivably in Pelvetia also, to supply the uronic acids for synthesis of polymers such as alginic acid (Bidwell and Ghosh, 1963; Lin and Hassid, 1966).

(c) The heterotrophic synthesis of mannitol.

In chapter 4 mannitol was shown to be the major soluble product of heterotrophic glucose metabolism in Pelvetia, but not in F. spiralis. Drew (1969) concluded that the monosaccharide reducing system of Fucus is photosynthetically linked. It nonetheless remains possible, since respiratory carbon loss by Fucus greatly exceeds glucose uptake, that reaction equilibria are so much in favour of mannitol respiration as to prevent polyol synthesis. It is possible that the failure of Fucus to produce mannitol heterotrophically

may also be a consequence of the different respiratory fates of glucose taken up by this species and by Pelvetia, and the consequent differences in coenzymes produced. When excised apical and basal portions of these species were incubated in media containing glucose-1-, -6-, or -U-^{14}C it was shown that in F. spiralis the P.Ph. cycle accounts for approximately 75% of glucose oxidation in apical tissue but only 10% in basal tissue. In Pelvetia the value was approximately 45% for both apical and basal tissues. Thus, F. spiralis will produce less NADH per mole of glucose oxidised than will Pelvetia, and this difference will be accentuated by the greater ability of the latter species to take up exogenously supplied glucose. Whether a NAD linked dehydrogenase is responsible for the heterotrophic synthesis of mannitol by Pelvetia is a point for conjecture.

Particulate preparations of these species were made according to the method of Lin and Hassid (1966), and incubations were conducted in the presence of glucose-U-^{14}C and NADP or NAD. Although evidence of respiration is provided, neither species produced mannitol in the presence of either coenzyme. Obviously at least one enzyme had been denatured during the extraction procedure. Under these in vitro conditions both F. spiralis and Pelvetia produced a compound which appears to have been free glucuronic acid. Little is

known of the fate of such acids in algal metabolism. D-mannuronic and D-guluronic acids were shown by Lin and Hassid (1966) to be the precursors of alginic acid. It is possible that glucuronic acid may enter the hexose-pentose (glucuronate-xylulose) cycle in Pelvetia and F. spiralis. No direct evidence for this cycle has been demonstrated for algae under in vivo conditions, although the apparent lack of G-6-P dehydrogenase and enzymes of the EMP pathway (Jacobi, 1958) suggests its operation in certain Rhodophyceae.

(d) Calculation of the pentose-phosphate pathway.

Initial calculations of the P.Ph. contribution in F. spiralis and Pelvetia were made using the formula of Katz and Wood (1960; 1963). The method was found to be inappropriate for these species in two respects;

(i) The specific activity of $^{14}CO_2$ released in respiration exhibits an intial lag but increases latterly at a proportionately greater rate than the specific activity of other compounds (alcohol soluble + insoluble fractions) which become labelled, and which in toto ($^{14}CO_2$ + alcohol soluble and insoluble fractions) represent the "depletion of media" used in calculating the specific yields as used by Katz and Wood. The specific yield is thus non-linear with respect to time. Drew (personal commurication) has shown this effect to be due to retention of some $^{14}CO_2$ in the sea-water buffer system.

(ii) Obviously, the theoretical ideal for such calculations is an infinitely short incubation period (which cannot be realised in practice) such that the recycling of hexose-P will be minimal. Because of recycling, calculated values for the P.Ph. cycle are expected to decrease with time. When correction is applied for recycling of hexose-P the calculated value for P.Ph. contribution should remain constant until the exogenously supplied glucose substrate is exhausted. Values calculated for the P.Ph. cycle by the method of Katz and Wood were not constant between 2 and 20 hours, after which time the medium was less than 50% depleted of glucose. Because of the initial lag in $^{14}CO_2$ released in respiration, as described in (i) above, the calculated values for P.Ph. cycle actually increased with time.

When absolute $^{14}CO_2$ yields were used the effect of isotopic non-steady state was removed as reference was not made to any other metabolite. By using the formula $G_p = \frac{(G_1 - G_6)}{G_U} \times 100\%$ the contribution of the P.Ph. cycle was recalculated from data obtained after 2 - 20 hours incubation. In accordance with theoretical expectation the values after 2 hours were higher than those after 15 or 20 hours, but only alightly so, suggesting that recycling of hexose-P is not extensive in these species.

3. Autotrophic and heterotrophic carbon fixation and loss.

Sieburth (1969b) demonstrated dark exudation of carbohydrate, nitrogenous and polyphenolic material from various intertidal algae. In F. vesiculosus this loss was coupled directly to photosynthesis, taking place only in the light. A carbon balance sheet for this species shows that approximately 40% of the net carbon fixed daily was lost by exudation. Considerable losses were also sustained during emersion, and lesser ones during rainfall or freezing.

(a) Heterotrophic uptake and loss of carbohydrate.

In the present study intact thalli of F. spiralis have been shown to exhibit considerable carbohydrate leakage on re-immersion in the dark. This leakage decreased with time showing the alga to be able to re-absorb much of the carbohydrate heterotrophically (figure 4:1b). Much of this re-absorbtion takes place within the first hour after re-immersion, but the remainder, approximately 150µg/100 mg. dry wt., must be taken into account when considering the efficiency of heterotrophic or autotrophic carbon fixation. It is likely that in the sea these losses are even greater because recovery (by the algae) from a large volume of medium is less likely ; Nalewajko (1962) has demonstrated that in Chlorella carbohydrate leakage increases as the medium:tissue ratio rises.

When using carbon-14 tracer techniques the values obtained for heterotrophic uptake of glucose in a closed system are possibly not absolute as some of the unlabelled and labelled endogenous carbohydrate will be exuded. The relationship between dark exudation and uptake is complex and net values for the latter can only be approximate when using radioactive tracers.

In chapters 4 and 5 uptake patterns of glucose-U-^{14}C into intact and excised thalli of F. spiralis and Pelvetia were demonstrated. In both of these species uptake into basal tissue was higher than that into apical tissue; this was particularly marked in the case of F. spiralis (table 4:1). When compared with respiratory carbon losses sustained during the same period it is evident that the metabolism of exogenously supplied glucose could not maintain growth in either of these species (table 9:2).

(b) Photosynthesis.

In chapter 8 photosynthetic carbon-14 fixation in Pelvetia apical tissue was studied under conditions of high and low light intensity, and in the presence or absence of exogenous glucose-^{12}C. The effect of glucose on photosynthesis is discussed in section 5 of this chapter.

Table 9:3 shows the photosynthetic carbon balance sheet for apical tissue of Pelvetia incubated in sea-water media containing $H^{14}CO_3^-$.

Table 9:2. The carbon balance sheet for dark incubated tissue of F. spiralis and Pelvetia.

	Heterotrophic C-uptake	Respiratory C-loss	Carbon deficit
	(µgC/100 mg. dry wt. / hr.)		
Pelvetia- excised tissue			
Apical	2.3	14.0	11.7
Basal	5.6	8.0	2.4
Pelvetia- intact tissue			
Basal	7.1	8.0	0.9
Fucus- excised tissue			
Apical	0.08	46.0	45.92
Basal	2.57	27.0	24.43
Fucus- intact tissue			
Apical	0.82	46.0	45.18
Basal	10.25	27.0	16.75

Note: (1) Data for heterotrophic uptake are taken from tables 4:1 and 5:1. Total uptake was divided by 15313cpm, which is equivalent to 1µg of glucose of the specific activity used.

(2) Data for respiratory loss are taken from table 3:3.

Table 9:3. The photosynthetic carbon balance sheet for excised apical tissue of Pelvetia.

Light intensity	Photosynthetic C-fixation	Respiratory C-loss	Net carbon + or -
	(µg C/100 mg. dry wt. / hr.)		
0.00143cal/min/cm² (16 ft. candles)	7.88	14.0	-6.22
0.245cal./min/cm² (2700 ft. candles)	402	14.0	+388

Note: (1) It is assumed that endogenous respiratory rate remains constant regardless of light intensity.

(i) Photosynthetic rate.

Under conditions of high light intensity apical tissue of Pelvetia fixed 388µg C/100mg. dry wt./hour

or 3880µg C/gm. dry wt./hour

The fresh weight:dry weight ratio for this species is approximately 20:1. Thus, 3880/20µg of carbon were fixed/hour per gm. fresh weight, or 194µg C/gm. fresh wt./hour.

Now, 12µg C = 44µg CO_2 = 22.4µl CO_2

Thus, 194µg C = 194 X 22.4/12µl CO_2

= 360µl CO_2/gm. fresh wt./hour.

Now, 1gm. fresh weight is equal to 10cm² of thallus.

Therefore, the rate of photosynthesis under these conditions is 37µl CO_2/cm²/hour, a rate close to the maximal value (33.3µl/cm²/hour) determined by Hardwick et al. (1968) for the annual angiosperm Perilla frutescens.

(ii) Compensation point.

When irradiated with 16 ft. candles (dull natural daylight) Pelvetia sustained a net carbon loss. Thus the compensation point for this species is somewhat greater than 16 ft. candles. Kandwisher (1966) determined a value of 10 ft. candles for Fucus (species not known).

Low compensation does not appear to be a major factor enabling these species to survive under conditions prevailing on the shore, where photosynthesis can proceed at maximal rates for only a few hours each day. As seen previously,

reduced respiration during emersion appears to be more important in this respect.

4. The carbon requirements of deep water algae; is heterotrophy ever really necessary?

Wilce (1967) has postulated that marine algae growing in deep water in the high arctic regions require a source of organic nutrition in addition to photosynthesis because there is relatively little light available to them at any time of the year.

What of the carbon balance sheet of such algae? By how much, if at all, does annual respiratory carbon loss exceed potential photosynthetic carbon fixation? Data taken from the literature enables such calculations to be made, at least approximately.

Respiration

Grainger (1959) and Lee (1966) have reported constant temperatures of -1.7°C in the high arctic. The effect that this temperature might have on respiratory rate is not known. Newell and Pye (1968) have shown that respiration in intertidal algae of boreal waters is relatively unaffected by temperature within the normal environmental range. Knadwisher (1966), working on Laminaria populations in Labrador, has suggested winter dormancy in arctic marine plants, whereas Kjellman (1877, 1883), working in Spitzbergen and Siberia,

observed reproductive growth in winter, suggesting metabolic activity. There is no quantitative data on the respiratory rates of arctic algae.

At temperatures below 0°C it is reasonable to expect respiration rates of the attached marine algae to be in the region of 0.2µl O_2/mg. dry wt./hour. This is equivalent to some 0.099µg. carbon lost/mg./hour (assuming mannitol to be the respiratory substrate). Assuming a biomass of 20gm. dryweight of algae/m^2 (Wilce gives no indication of this value, but states that the arctic biomass is small relative to that of boreal waters), it would be expected that the ecosystem lost some

$$\frac{0.92.\ 0.20.\ 12.\ \ 24.\ 365.\ 20.\ 10^3}{22.4.\ \ 10^6} \text{ gm. carbon lost/m}^2\text{/year.}$$

= 18.3 gm. carbon/m^2/year lost in respiration.

Photosynthesis

The data shown in table 9:4 for incident mean daily light energy and its transmittance through the ice are taken from Wilce (1967, table 1) and are the results of Lee (1966). The extrapolated data for light energy at 20m. are taken from Wilce (1967, figure 1). It should be noted that the assumptions which follow are by and large extreme and should tend towards the credibility of Wilce's hypothesis.

Assuming Lee's data for re-reflected light (75%), then the fraction which enters the snow is 0.25. Assuming

also that there is average or heavy snow cover above the ice (extinction coefficient 0.011/cm.) then the light energy transmitted through the ice is;

0.25 X incident energy X snow transmittance X ice transmittance

Wilce states that maximal growth of attached algae is deeper in arctic than in boreal waters, being 10-25m. below the surface. Let us assume an average of 20m.

In his figure 1. Wilce shows extinction coefficients for light passing through arctic water in June, July and August. The average light attenuations in these months at 20m. are 55%, 78% and 80% respectively. As the season progresses turbidity increases, so let us say there is 85% attenuation in September. In terms of light energy these months from June to September are the only quantitatively significant ones.

Under these conditions it is possible to calculate (table 9:4) that under average snow cover at 20m. a total illumination of some 1980 Kcal/m^2/year is available to the plants for photosynthesis (excluding November, December and January). This energy is calorimetrically equivalent to 660gm. of dry organic matter or 330gm. of carbon. Similarly, for heavy snow cover it can be calculated that the light energy available is equivalent to 190gm. of carbon.

As seen in table 9:4 annual photosynthetic fixation at 20m. would always exceed respiratory loss down to

Table 9:4. Annual light energy at 20m. in the arctic sub-ice environment.

month	incident mean daily light at surface	transmitted light beneath ice heavy	beneath ice average	at 20m. heavy	at 20m. average	Kcal/m² monthly at 20m. heavy	Kcal/m² monthly at 20m. average
Feb.	120	0.03	0.04	0.03	0.04	0.84	1.1
March	1100	0.11	0.22	0.11	0.22	3.4	6.8
April	2100	0.08	0.30	0.08	0.30	1.2	9.0
May	2600	0.24	3.9	0.24	3.9	7.4	121
June	2850	1.02	9.4	0.46	4.2	1.4	126
July	2350	28.5	63.5	6.3	14	195	435
Aug.	1560	106	116	21.2	32	660	990
Sept.	730	54	54	8.1	8.1	245	245
Oct.	180	6.0	9.0	0.9	1.5	28	46.5
Total	13590	196	257	37	65	1142	1980

Note: (1) Incident mean daily light at surface and transmitted light are expressed as Kcal/m²/day.

(2) "heavy" and "average" refer to the depth of snow.

(3) 1 ft. candle = 0.6 ergs/sec/mm² = 1.3 Kcal/m²/day.

photosynthetic efficiencies of 10%. Extrapolating to 50m., where light energy is about 20% that at 20m. (but where the biomass is also reduced), available light energy would be 395 or 230 Kcals/m^2/year beneath average and heavy snow cover respectively. These values are equivalent to 66 and 38gm. carbon respectively, probably allowing for net carbon accretion, at least under average depths of snow, at 25% efficiency. Although values of as much as 10% efficiency are rare in land plants, recert work has indicated that under conditions of very dim light marine algae may be greater than 50% efficient (Drew personal communication).

In these calculations no reference has been made to those algae growing beneath a thin snow cover, through which transmittance is somewhat higher (Lee, 1966), making more light available for photosynthesis.

Equally, however, the important question of compensation point has not yet been raised. Wilce has based his hypothesis on the compensation point (8 ergs/sec/mm^2) determined by Haxo and Blinks (1950) for Porphyra naiadum, a red alga of the North American Pacific coast. Bunt (1964) has shown the under-ice antarctic phytoplankton association to reach its compensation point at 3 ergs/sec/mm^2. Wilce concludes that "with the very low amount of energy available to deep sublittoral arctic algae, these plants would seem

Table 9:5. Photosynthetic carbon fixation at 20m. in the arctic sub-ice environment, under average snow cover.

photosynthetic efficiency %	theoretical photosynthetic C-equivalence (gm. C per year)	C-fixation above compensation (gm. C per year)
100	330	180
75	275	135
50	165	90
25	83	45
10	33	18

to need compensation points even lower than any yet known - if they are strictly autotrophs". However, allowing for a compensation point at 3 ergs/sec/mm^2 it can be calculated that algae growing at 20m. beneath ice and an average snow cover receive some 1071 Kcal/m^2/year, equivalent to 18 gm. of carbon fixed photosynthetically at an efficiency of only 10% (table 9:5). This is close enough to the assumed respiratory losses, in view of the approximations involved in these calculations, to suggest that the plants, although necessarily slow growing, may survive by purely autotrophic nutrition.

5. The stimulation of photosynthesis by exogenously supplied glucose.

In chapter 8 it was shown that in the presence of exogenous glucose (0.2mM) carbon fixation was enhanced at low and high incident light intensity by factors of 20% and 43% respectively. This stimulation can be accounted for either in terms of non-photosynthetic carboxylation, or the alteration of an extrinsic rate limiting factor in photosynthesis. These alternatives are outlined below.

(a) The operation of another pathway in addition to photosynthesis; for example, by the carboxylation of a glycolytic derivative of glucose-^{12}C, such as P-enolpyruvate (by the PEP carboxylase reactions of Bandurski and Greiner, 1953, and Kurahasi et al., 1957) or pyruvate (the Wood-

Werkman reaction of Suzuki and Werkman, 1958, and Ochoa et al., 1948; or the malic enzyme reaction of Rutter and Lardy, 1958).

Although the enzymic reactions responsible for such dark reactions have not been investigated in algae, the sequence of labelling patterns in Chlorella, as elucidated by Moses et al. (1959) indicates the likelihood of their occurrence in this alga.

This process, however, would be limited by the rate at which the heterotrophic uptake of glucose-^{12}C could proceed. If, as postulated, 3-C units were responsible for the pick-up of $^{14}CO_2$, then carboxylation could only proceed at a maximal rate equal to twice that heterotrophic 6-C uptake. Also, a similar increase in dark fixation of $^{14}CO_2$ would be expected to occur (in the presence of glucose-^{12}C) if this system were operative; as seen previously (table 8:1) this was not the case. For these reasons it seems unlikely that this method could account for the observed enhancement of photosynthesis in the presence of glucose-^{12}C.

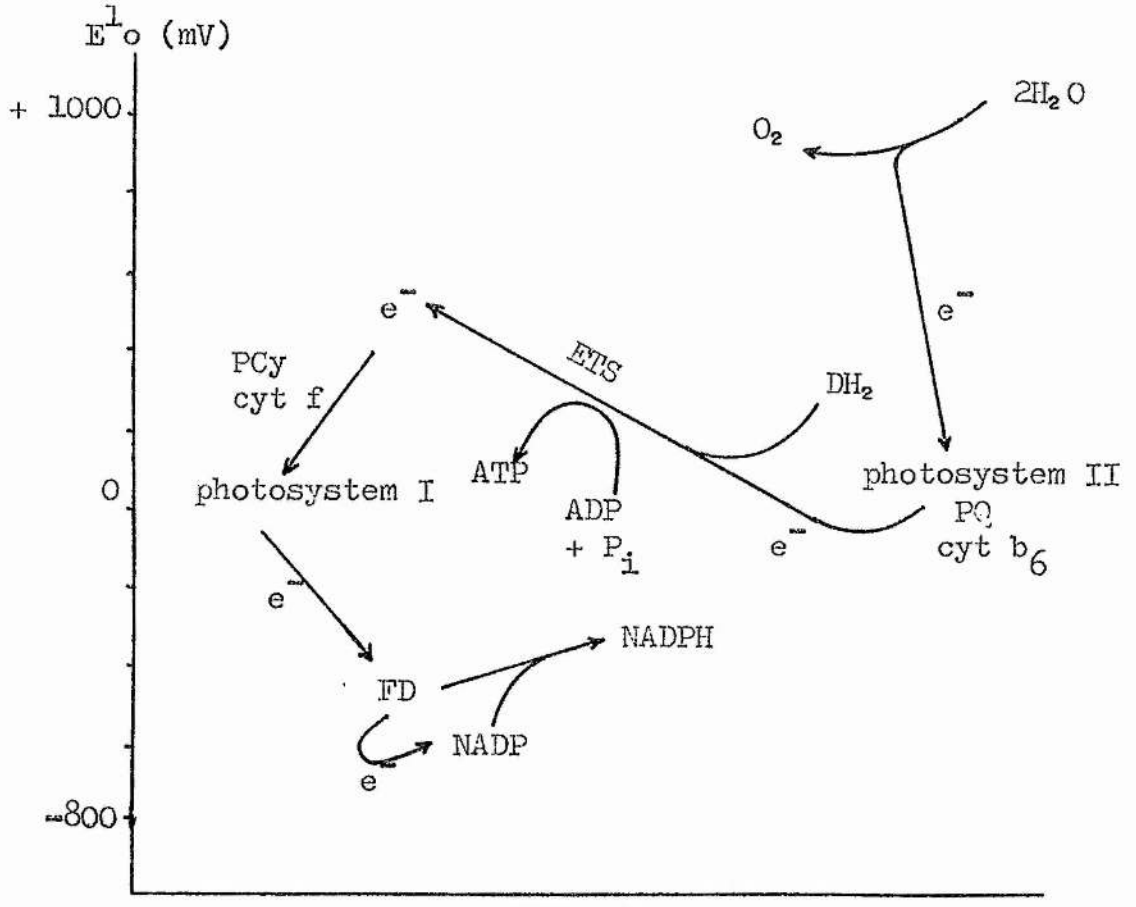
(b) Alternatively, glucose may be affecting the rate of photosynthesis per se; two alternative explanations whereby this might happen are offered here.

(i) That CO_2 was initially rate limiting and that glucose-^{12}C was metabolised to an acid, which, since

the interior of the cell is electronegative, was actively excreted, thus lowering the pH of the medium (or at least, preventing the normal pH increase concommitant with photosynthesis). This would serve to increase the availability of free CO_2 for photosynthesis. Certainly, no increase in pH of the medium was observed after 2 hours, but equally there is no evidence that CO_2 was initially rate limiting, although a diffusion pathway of 0.5 mm. (max.) might suggest this to be so. Indeed, it is most unlikely that CO_2 was rate limiting at the low light intensity (light energy approximately 0.25% that of diffuse daylight energy), thus suggesting that the photochemical reactions were rate limiting. This could be tested critically by enhancing the CO_2 content of the sea-water medium.

(ii) It is possible that the photochemical processes were rate limiting (light energy of low and high light intensity treatments was equivalent to approximately 0.25 and 33% respectively of diffuse daylight intensity). In this case one must postulate that the enhancement of photosynthesis was due to the use of glucose as a reductant, possibly entering the Hill and Bendall 'Z schema' (Hill and Bendall, 1960) after photosystem 11 (figure 9:1). Such a system is operative in the Athiorhodaceae(Van Niel, 1941), and has been induced in bacteria and higher plants by the use of reduced phenazine methosulphate ($<10^{-4}$M), and reduced dichlorophenolindophenol ($<10^{-5}$M), donors capable of feeding

Figure 9:1. The scheme for two photochemical reactions in photosynthesis.



Abbreviations:

e^-	electron
PQ	plastoquinone
cyt b_6	cytochrome b_6
ETS	electron transport system
PCy	plastocyanin
cyt f	cytochrome f
FD	ferredoxin
DH_2	a donor capable of feeding electrons that generate phosphorylation, thus obviating the requirements for photosystem II.

electrons that generate phosphorylation, thus obviating the requirement for photosystem 11. A similar conclusion, that organic substrates can act not only as sources of carbon, but also as H-donors for photoreduction, was reached by Vishniac and Reazin (1957) working on Ochromonas malhamensis.

To test if glucose is acting as a reductant one would require to block photosystem 11 (for example, using circa 10^{-7}M 3-(3,4-dichlorophenyl)-1,1-dimethyl urea, DCMU), in which case carbon fixation would be expected to proceed at a rate equal to the observed enhancement effect induced by exogenous glucose. Other evidence which would strongly support this hypothesis would be if oxygen production were negated in the presence of DCMU, and further, if oxygen outputs in treatments with or without the exogenous metabolite were found to be equal.

It is felt that the present evidence is strongly in favour of the latter explanation, but whatever explanation should prove to be valid the important point is that the presence of exogenous glucose reduces the light requirement for photosynthesis. Should other organic metabolites found freely in the sea also prove to exhibit this effect, this would be of considerable ecological significance.

Furthermore, the system offers a reasonable

alternative to the hypothesis of heterotrophy, which finds little experimental support in the species under present investigation.

CHAPTER 10

Summary

The upper littoral brown macrophytic algae Pelvetia canaliculata and Fucus spiralis both contain mannitol, and Pelvetia contains similar amounts of a second polyol, volemitol. In summer the mannitol content of both species was greater in apical than in basal tissue, whilst in winter the basal content was increased and the apical content depleted (to a lower value than that for basal tissue in F. spiralis). Volemitol decreased in both apical and basal tissue during the winter. The fluctuation in mannitol content is consistent with its production in photosynthesis and its loss as a respiratory substrate. Why volemitol (which otherwise appears to be metabolically inactive) should show annual fluctuation is not clear.

It has not been possible to substantiate the hypothesis that polyols might serve as endogenous osmo-regulators. It appears that high levels of endogenous polyols in algae grown in saline media are not themselves responsible for increased osmotic pressure, but are a consequence of it. This is consistent with the proposed role of polyols in the control of metabolic pathways by the regulation of co-enzyme levels.

Absolute yields of CO_2 released in the respiration of exogenously supplied isotopes of glucose have been used in the estimation of the pentose-phosphate pathway. The results from short and long term incubations suggest that recycling of hexose-P is not extensive in these species. It appears, then, that the major function of this cycle in these species is the production of 5-carbon units, and as these do not appear as pentitols or pentoses it is probably reasonable to assume that active nucleic acid synthesis takes place under the conditions of these experiments.

From the data available it is not possible to indicate how mannitol is synthesized heterotrophically by Pelvetia, or indeed why it is not synthesized by Fucus under the same conditions. Several reasons have been offered to explain why mannitol synthesis should be photosynthetically linked in Fucus:

(a) Pelvetia may be more permeable to exogenous glucose than is Fucus. Conversely, the rate of glucose metabolism may control its rate of uptake. This could be tested critically by the use of 3-O-methyl glucose which is taken up but not metabolised.

(b) Respiratory carbon loss by Fucus greatly exceeds glucose uptake; reaction equilibria are possibly so much in favour of mannitol catabolism as to prevent its simultaneous synthesis.

(c) It is possible that the necessary co-enzyme for mannitol synthesis is preferentially channelled into other reductive reactions.

Particulate preparations of the algae did not further clarify this situation. They did, however, indicate the synthesis by both species of glucuronic acid, the first product of the hexose-pentose cycle, of which the pentose phosphate pathway is an integral part. Whether these cycles are linked *in vivo* is dubious as the ratio of $^{14}CO_2$ from glucose-6-C^{14} to that from glucose-1-^{14}C was never found to exceed unity.

The heterotrophic uptake and metabolism of glucose by *Pelvetia* and *Fucus* shows several interesting contrasts. In *Pelvetia* the process is relatively rapid and continuous, whereas in *Fucus* the process is somewhat slower and quite rapidly saturated. The major soluble product in *Pelvetia* is mannitol; in *Fucus*, non-metabolised glucose is the major soluble component, although a second, unidentified, product has also been detected. The ability to take up exogenously supplied glucose and to retain it shows a regional variation along the thalli in these species. This is particularly marked in *F. spiralis*, in which basal tissue takes up more than one hundred times the amount of glucose than does apical tissue. No translocation of this or other sugar or polyol has been evidenced in either species. This is possibly a reflection of the relative endogenous carbohydrate

pool sizes of apical and basal tissue, but it is nonetheless consistent with the now widely held belief that trumpet hyphae are non-conductive.

In both Pelvetia and F. spiralis the respiratory rate is reduced during emersion. Reduced gaseous exchange during emersion could be extremely significant in intertidal algae in that respiratory substrate is thereby conserved.

In the presence of exogenous glucose no increase in respiratory rate was evident; whether this can be attributed to hexokinase deficiency is uncertain. Respiratory rate during immersion, like glucose uptake, is variable along the thalli of these species, declining from apex to base. The rate in Pelvetia is about half that in F. spiralis, which again could be of ecological significance. In both these species, however, the respiratory carbon loss is greater than the heterotrophic uptake (at least of glucose). In Pelvetia basal tissue, however, the rate of uptake closely approaches the rate of carbon loss such that during periods of dark immersion the status quo may be maintained by heterotrophy, but without net growth. It is doubtful, however, whether levels of exogenous glucose in the sea approach 0.2mM, the concentration used in these experiments.

Studies of photosynthesis in Pelvetia have shown that the photosynthetic rate of this species is high, being similar

to that of the annual angiosperm Perilla frutescens. The high compensation point (>16 ft. candles) is obviously not contributory to this high rate of photosynthesis. In view of these facts it is likely that the quantum efficiency of photosynthesis will be high in Pelvetia. Under the same conditions of incident illumination it has been shown that the presence of exogenous glucose enhances photosynthetic carbon fixation by up to 43%, an amount considerably greater than simultaneous photoassimilative fixation. Assuming that the photochemical reactions of photosynthesis were rate limiting then this effect can be attributed to the use of glucose as a reductant, possibly feeding electrons that generate phosphorylation. The significance of this enhancement effect is that in the presence of glucose the light requirement for photosynthesis is reduced. Should other metabolites found freely in the sea be similarly used by this and other species the resultant increase in photosynthetic efficiency might play an important role in the survival of algae wherever light is rate-limiting on photosynthesis. It is felt that a reduced light requirement for photosynthesis offers a reasonable alternative to the hypothesis of heterotrophy which finds little experimental support in the species under present investigation.

Conclusions

1. Pelvetia canaliculata and Fucus spiralis both contain mannitol, but volemitol is found only in Pelvetia. No free sugars have been found in either species. Mannitol appears to be the respiratory substrate. No metabolic role has been designated to volemitol in the present study. Both polyols are produced photosynthetically, and their total contents are higher in summer than in winter.
2. Respiratory CO_2 loss is impaired by approximately 75% in emersed tissue of Pelvetia.
3. Considerable carbohydrate losses are sustained by F. spiralis on immersion, but not by Pelvetia.
4. Immersed respiratory carbon loss from F. spiralis is approximately double that from Pelvetia, and in both species apical respiration is greater than that of basal tissues.
5. D-threo-chloramphenicol (CAP) uncouples oxidative phosphorylation and, as a secondary effect, reduces uptake of exogenous glucose. This is considered to be due to decreased ATP availability and suggests that glucose uptake might normally be active in F. spiralis.
6. In the presence of exogenous glucose Pelvetia is able to synthesize mannitol heterotrophically, F. spiralis is not. Apical tissue of F. spiralis takes up very little glucose; most of this is elutable and 50% of that which remains

is bound to cell wall material. Glucose uptake by basal tissue of F. spiralis is comparable to that in Pelvetia.

7. There is no evidence for the translocation of labelled glucose or its metabolic products.

8. Apical tissue of F. spiralis respires exogenously supplied glucose largely via the pentose-phosphate pathway. Little P.Ph. cycle activity is evidenced in basal tissue of this species. The P.Ph. cycle is equally active (about 40%) in apical and basal tissues of Pelvetia. In both species there is little evidence for recycling, the P.Ph. cycle apparently acting as a shunt.

9. Particulate preparations of Pelvetia and F. spiralis both synthesized glucuronic acid from glucose. In vivo activity of the hexose-pentose cycle in these species is dubious.

10. Available information indicates that heterotrophic metabolism (at least of glucose) could not support net growth in either Pelvetia or F. spiralis, and further, that such supplementary carbon fixation cannot even account for respiratory carbon loss.

11. Re-appraisal of Wilce's data for algae growing in the Arctic sub-ice environment suggests that it is also unnecessary to postulate heterotrophic supplement of the autotrophic processes in these algae.

12. The photosynthetic rate of Pelvetia has been shown to be high. This cannot be accounted for in terms of light compensation, which is also high. It has been suggested

that exogenous glucose lowers the light requirement for photosynthesis by acting as a reductant, thereby feeding electrons that generate phosphorylation.

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